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(54)【発明の名称】歯周病原性菌由来酵素およびその測定方法並びに当該酵素に対する抗体

(57)【要約】 (修正有)

【構成】 次の酵素学的性質を有する、ポルフィロモナス・シンジバリス由来酵素およびその測定方法並びに当該酵素に対する抗体。

(1) 作用：歯周組織に対する分解能を有し、また炎症性細胞に対する障害活性を有する。

(2) 基質特異性：コラーゲン、免疫グロブリンなどの各種蛋白質に対して高い分解能を有し、合成蛍光基質対し特異的に強い分解活性を示す。

(3) pH：至適pHは7~8、安定pH4~9

(4) 作用適温の範囲：室温から37°C

(5) 分子量：ゲル汎過で約50kDa、SDSゲル電気泳動で約44kDa

(6) 等電点：pH5~5.5

【効果】歯周病の進行状況と活動度を経験に依存することなく的確に把握でき、酵素に対する抗体は、分析用試薬の他、歯周病の予防、治療剤としての利用も期待できる。

## 【特許請求の範囲】

【請求項1】 以下の酵素学的性質を有する、ポルフィロモナス・ジンジバリス (*Porphyromonas gingivalis*) 由来酵素：

(1) 作用；コラーゲンを主体とする歯周組織に対し直接的な分解能を有するとともに、好中球などの炎症性細胞に対して障害活性を有する。

(2) 基質特異性；コラーゲンや免疫グロブリンなどの各種蛋白質に対して高い分解能を有する一方、合成蛍光基質セーブチルオキシカルボニル-L-フェニルアラニル-L-セリル-L-アルギニン-4-メチルクマリル-7-アミド (Boc-Phe-Ser-Arg-MCA) およびカルボペニゾイル-L-フェニルアラニル-L-アルギニン-4-メチルクマリルアミド (Z-Phe-Arg-MCA) に対し特異的に強い分解活性を示す。

(3) 至適pH及び安定pH；至適pHは蛋白質基質及び合成基質のいずれの場合も7~8にあり、pH4~9の範囲で安定である。

(4) 作用適温の範囲；室温から37°C

(5) 活性化；システイン、2-メルカプトエタノール、ジチオスレイトールなどのSH基還元剤によって著しく活性化される。

(6) 阻害物質；キモスタチン、ロイベプシン、E-64、アンチバイン、EDTA、TPCK、TLCCKなどにより強い活性阻害を受ける。

(7) 分子量；ゲル汎過で求めた見かけ上の分子量は約50kDa、SDSゲル電気泳動で決定した分子量は約44kDaである。

(8) その他；等電点pH5~5.5

【請求項2】 以下の工程を有することを特徴とする請求項1記載の酵素の測定方法：

(1) 試料の一部を、EDTA及びロイベプシン存在下で請求項1記載の酵素の基質と反応させ、酵素活性を測定する工程。

(2) 当該試料の一部に請求項1記載の酵素に対する特異抗体を作用させた後、当該反応液を固相と液相に分離し、次いで上記(1)に従って酵素活性を測定する工程。

(3) 工程1の酵素活性より工程2の酵素活性を差引き、請求項1記載の酵素活性量を算出する工程。

【請求項3】 特異的に請求項1記載の酵素と結合する抗体。

【請求項4】 次の(a)~(d)

(a) 特異的に請求項1記載の酵素と結合する抗体、  
(b) 請求項1記載の酵素に特異的に認識される基質、

(c) ロイベプシンおよび

(d) EDTA

を含むことを特徴とする歯周病原性菌由来酵素測定用キット。

## 【発明の詳細な説明】

## 【0001】

【産業上の利用分野】本発明は歯科における重要疾患、とくに歯周病の診断および治療において有用な歯周病原性菌由来酵素およびその測定方法並びに当該酵素に対する抗体に関する。

## 【0002】

【従来の技術】歯周病は今日の高齢化社会において歯科領域の最も重要な疾患となっている。一生涯、自分の歯でものを食べたいという欲求が益々強くなる一方で、歯の喪失を引き起こす歯周病は確実に年齢とともに増え続けている。歯周治療を進める上で病態を把握する歯周診査ならびに診断は極めて重要である。

【0003】現在、歯周診査や診断に使用されている臨床パラメーターとしては(1)歯周ポケットの深さ (probing depth) や付着の喪失量 (attachment loss)、(2)歯の動搖度、(3)歯肉の炎症状態を現す指数 (gingival index)、(4)歯垢の累積状態を現す指数 (plaque index)、(5)出血を現す指数 (gingival bleeding index)、(6)X線写真からの歯槽骨吸収状態、(7)歯肉溝の滲出液量 (GCF volume)などの項目が知られている。

【0004】しかし、これらの臨床パラメーターは以下の点で問題を残しており、十分に満足の行くものではなかった。まず、歯周ポケットの深さや付着の喪失量の測定やX線写真からの歯槽骨吸収の程度の判定などは、歯周病の最も重要な病態である歯周組織の破壊の程度を知るための有用な臨床パラメーターとして汎用されているが、これらはあくまでも過去の炎症による歯周組織破壊の結果を示すものであり、現状における歯周組織破壊の活性度や歯髄などへの影響を知る手掛かりとはなり得ない。

【0005】また、歯肉指数 (gingival index)、歯垢指数 (plaque index)、出血指数 (gingival bleeding index)、歯の動搖の程度および歯肉溝の滲出液量 (GCF volume)などは現今の病状を反映するパラメーターであるが、判定基準が極めて大まかであったり、特殊な測定装置を必要とする (GCF volume) など、複雑なステップで進行する歯周炎の現状の活動度の診断や治療の必要性を判定するための臨床パラメーターとしては正確さや再現性、客観性に欠ける難点があった。このように、歯周病の診断等において客観的な診査方法や正確な診断方法等はないのが現状であり、これは当該分野において世界的な問題となっている。

【0006】最近、歯周病原性菌のひとつであるポルフィロモナス・ジンジバリス (*Porphyromonas gingivalis*) 由来の複数の酵素を適当な基質及び/又はアクチベーターを用いて測定することにより歯周病の診断を行う方法が見いだされているが (国際出願公開公報; WO 92/07086)、当該発明方法では酵素の特定がなされていないことや、測定に用いる基質の特異性や感度が

低いなどの欠点があり、さらにこれらの方で測定される酵素活性が生体由来の各種インヒビター (endogenous inhibitors such as serpins and cystatins) でどの程度影響を受けるのか不明であり、これらの酵素がインヒビターによって影響されれば、当該発明方法で測定された酵素量は正確なものとはいはず、当該方法は歯周病の診断に用いるには未だ不十分なものであった。

## 【0007】

【発明が解決しようとする課題】歯周病の診断等に関して、当該病状の進行状況を客観的に知ることは、その後の治療をどのように行うかを判断するに際して非常に重要であるが、上記のように未だ十分に満足できる方法が提供されておらず、歯周病の進行状況を簡便に判断できる方法の開発が切望されていた。

## 【0008】

【課題を解決するための手段】本発明者らは、歯周病原性菌のひとつである *P. gingivalis* の歯周病における作用について検討していたところ、当該菌体がプロテアーゼである新規な酵素を産生しており、この酵素が歯周病に関与していることを見いだした。

【0009】また本発明者らは、歯周病の病状の進行に比例して歯周病臨床患者の歯肉溝滲出液中で上記酵素活性が上昇し、歯周病の病勢と当該酵素の活性上昇の程度に相関関係があるという新たな知見を見いだした。

【0010】特に、当該酵素は歯周組織の主要成分であるタイプIコラーゲンを強く分解するコラゲナーゼ活性を有するなど、歯周組織の直接的破壊を引き起こすことや好中球などの炎症性細胞に対して機能障害を引き起こし生体防御系を破壊するなど、歯周病の発症や進行と密接に関係する性質や機能を有することが明らかとなり、従来より知られている *P. gingivalis* 由来の酵素を測定するよりも当該酵素を測定することの方が歯周病の病状の進行を知る上で非常に有意であるということを見いだした。

【0011】本発明は、上記知見に基づき完成されたものであり、*P. gingivalis* の産生する前記酵素およびこの酵素の検出方法を提供するものである。

【0012】本発明の酵素は、*P. gingivalis* に属する微生物を利用し、例えば、以下のようにして得ること

ができる。まず、*P. gingivalis* に属する微生物を培養し、その培養上清に硫安を加えて70%飽和とした後、生じた沈殿を遠心分離等の手段によって集め、これを、例えば非イオン性界面活性剤を含むリン酸緩衝液に対して透析する。次いで、遠心透析上清をリン酸緩衝液等で平衡化したDEAEセファセル等のカラムにかけ、得られる非吸着分画を濃縮し、さらにCMトヨバル等のカラムにかけ、活性画分を溶出する。最後に、溶出画分を濃縮、透析した後、pH 3.5~10の範囲の等電点分離にかけ、pH 5.0~5.5の活性画分を集め、更に濃縮、透析後、TSKゲルG2000SW等のゲル沪過に付すことにより精製酵素として得ることができる。

【0013】斯くして得られる本発明の新規酵素（プロテアーゼ）は以下に示すような特異的な酵素学的性質を持つ。

【0014】(1) 作用；コラーゲンを主体とする歯周組織に対し直接的な分解能を有するとともに、好中球などの炎症性細胞に対して障害活性を有する。この酵素の分解様式は比較的の非特異的であり、エンドプロテナーゼ (Endoproteinase) として作用する。

(2) 基質特異性；コラーゲンや免疫グロブリンなどの各種蛋白質に対して高い分解能を有する一方、合成蛍光基質 *t*-ブチルオキシカルボニル-*L*-フェニルアラニル-*L*-セリル-*L*-アルギニン-4-メチルクマリル-7-アミド (Boc-Phe-Ser-Arg-MCA) およびカルボベンゾイル-*L*-フェニルアラニル-*L*-アルギニン-4-メチルクマリルアミド (Z-Phe-Arg-MCA) 等を特異的に分解するアルギニルエンドペプチダーゼ (arginyl endopeptidase) 活性を有する。

【0015】(3) 至適pH及び安定pH；図1に示すように、至適pHは蛋白質基質及び合成基質のいずれの場合も7~8にあり、pH 4~9の範囲で安定である。

(4) 作用適温の範囲；室温から37°Cである（図2参照）。

【0016】(5) 活性化；表1に示すように、システイン、2-メルカプトエタノール、ジチオスレイトールなどのSH基還元剤によって著しく活性化される。

表 1

チオール化合物	濃 度 (mM)	残存活性 (%)
なし システィン	—	100
	1	7760
	5	8530
	10	7690
2-メルカブトエタノール	1	7290
ジチオスレイトール	1	8410

【0017】(6) 阻害物質; 表2に示すように、キモスタチン、ロイペプチニ、E-64、アンチパイン、EDTA、TPCK、TLCKなどにより強い活性阻害

を受ける。しかし、シスタチングループ（卵白シスタチンやヒトシスタチンSなど）では全く影響を受けない。

表 2

化 合 物	濃 度	残存活性 (%)
なし	—	100
キモスタチン	50 μg/ml	2
TPCK	1 mM	5
TLCK	1 mM	20
PMSF	1 mM	76
エラスタチナール	50 μg/ml	83
DFP	1 mM	111
ロイペプチニ	50 μg/ml	0
E-64	50 μg/ml	4
アンチパイン	50 μg/ml	7
ヨード酢酸	1 mM	33
卵白シスタチン	50 μg/ml	118
ヒトシスタチンS	500 μg/ml	125
ペプスタチン	50 μg/ml	112
EDTA	1 mM	18
EGTA	1 mM	45
ホスホラミドン	1 mM	46
CaCl <sup>2</sup>	1 mM	133
MgCl <sup>2</sup>	1 mM	139
FeCl <sup>3</sup>	1 mM	87
ZnCl <sup>2</sup>	1 mM	68

【0018】(7) 分子量; ゲル汎過で求めた見かけ上の分子量は約50 kDa、SDSゲル電気泳動で決定した分子量は約44 kDaである。

(8) その他; 等電点 pH 5-5.5

【0019】そして上記酵素は、下の式で示される構造

またはこれと類似するペプチド配列を有すると推定される。なお、式中には当該ペプチド配列をコードする塩基配列も併せて示した。

【0020】

【化1】

TTTAATGCTATAAAATACAGAAGGGTACTACACAGTAAAATCATATTCTAATTTCATCAA  
 ATGAAAAAACITGAAACAAGTTGTTGATGCTTCTTGCTCTTCCTTATTAAGGAGGAATG 60  
 M N L N K F V S I A L C S S L L G G M 20  
 GCATTGCGCAGCAGACAGAGTTGGGACGCAATCCGANTGTCAAGATTGCTCGAATCCACT 20  
 A F A Q Q T E L G R P N V R L L E S T 40  
 CAGCAATTCGGTGCACAAAGGTTCACTTCCCTATGGACAACTCTCAAGTTCACCGAAGTCAA 240  
 Q Q S V T K V Q P R M D N L F T E V Q 60  
 ACCCCCTAAAGGAATGGCACAAAGTCCCGACCTATACAGAAGGGGTTAAATCTTCCGAAAAAA 300  
 T P K G M A Q O V P T Y T E G V N L S E K 80  
 GGGATGCCCTACGCTTCCOCATTCTATCAOGCTTCTGGCGTTTCAGACACTCGTGAGATG 360  
 G M P T L S R G S D T R E M 100  
 AAGGTAGAGGTGTTCTCAAAGTTCATCGAAAAGAAAATGTCCTGATGTCACCCCTCC 420  
 K V E V V S S K F I E K K N V L I A P S 120  
 AAGGGCATGATTATGCGTAACGAGATCCGAAAAAGATCCCTTACGTTTATGCGAAGAGC 480  
 K G M I M R N E D P K K I P V V Y G K S 140  
 TACTCGCAAAACAATTCCTTCCGGGAGAGATGCGCACGCTTGTATGATCCTTCTTCTT 540  
 Y S Q N K F F P G E I A T L D D P F I L 160  
 CGTGATGTCCTGACACGGTTGTAACCTTTCGCTTTCAGTATAACOCTGTGACAAAG 600  
 R D V R G Q V V N F A P L Q Y N P V T K 180  
 ACGTTGCGCATCTATACGGAAATCACTTGGCAGTCAAGGAAACTTGGGAACAAGGCAA 660  
 T L R I Y T E I T V A V S E T S E Q K 200  
 AATATTCGAAACAGAAGGTACATTTCGGCTTTGAGACACATACAACGGCATGTC 720  
 N I L N K X G T F A G F E D T Y K R M P 220  
 ATGAACTACCGAGCGGGGGCTTACACACCGGTAGAGGAAAACAAAATGGTGTATGATC 780  
 M N Y E P G R Y T F V E E B K Q N G R M I 240  
 GTCATCGTAGCCAAAAGGATATTAGGAGATTTAAAGATTTCGTTGATGGAAAACCAA 840  
 V I V A R K Y E G D I K D F V D W K N Q 260  
 CGCGCTCGTACCGAGGGTGAAGTGGCAGAAGATATTGCTTCTCCGGTTACAGCTAAT 900  
 R G L R T E V K V A E D I A S P V T A N 280  
 GCTTATTCAGCAGTTCTTGTACCGAGAAATACCGAGAAGAGCTAATGATTTGACCTATGTT 960  
 A I Q O F V K Q E Y K E G N D L T Y V 300  
 CTTTGTTGGCAGTCACAAAGATAATTCTGCCRARATFACTTCGGGGATCAAATCCGAC 1020  
 L L V G D H K D I P K I T P G I K S D 320  
 CAGGTATATGGACAATAGTAGGTAAATGACCAACTACAACGAAGTCTTCATGGTGTTTC 1080  
 Q V Y G Q I V G N D H Y N E V F I G R F 340  
 TCAATGAGAGCAAAGAGGATCTGAAAGACACAAATCGATGGACTATTCACTATGAGCGC 1140  
 S C E S K E D L K T Q I D R T I K Y E R 360  
 AATATAACCACCGAAGACAAATGGCTGGTCAAGGCTTCTGTTGATTCGGCTGAAGGA 1200  
 M I T T E D K W L G Q A L C I A S A E G 380  
 GGCCCATCCGAGACAATGGTAAAGTGTATTCAGCATGAGAAATGTAATGCCAATCTG 1260  
 G P S A D N G E S D I Q H E N V I A N L 400  
 CTIACCCACTATCGCTATACCAAGATTTCAAACTTGTATGATCCGGAGTAACTCTTAA 1320  
 L T Q Y G Y T K I I K C Y D F G V T P K 420  
 AACATTAATGATGCTTCAACGGGAGATCTGGTGGTCAACTATACGGGCCACGGTAC 1380  
 N I I D A F N G G I S L V N Y T G H G S 440  
 GAAACAGCTTGGGGTACGTCTCACTTCGGCACCACCTATGAAAGCAGCTTACCAACACC 1440  
 E T A H G T S H F G T T H V R Q L T N S 460  
 AACCCAGCTACCGTTTATTCGAGCTGAGCTCTGAGAAGGGGATTTCTTATTCAGCATG 1500  
 N Q L P F I F D V A C C V N G D F L F S N 480  
 CCTTGCTTCGAGAAGCCCTGATGCGTGCACAAAAGAGATGTAAGCGCAGAGGTACTGTT 1560  
 P C F A E A L M R A Q K D G R P T G T V 500  
 GCTATCATACGGCTTACGATCAACCACTCTGGCTTCTCCATAGCGCCGGCAGGATGAG 1620  
 A I I A S T I N Q S W A S P M R G Q D E 520  
 ATGAAAGAAATCTGTGCGAAAACACCCGAAACACATCAAGCGTACTTTCGGTGTTGTC 1680  
 M N E I L C E R H P N N I K R T F G G V 540  
 ACCATGAACGGTATGTTGCTATGGTGGAAAAGTATAAAAAGGATGGTGGAGAGATGCTC 1740  
 T M N G M F A N V E K Y K K D G E K M L 560

【化2】

GACACATGGACTTTTCGGCACCCTCGCTGCTGGTGTACACTTGCCGACCAAA 1800  
 D T W T V F G D P S L L V R T L V F T K 580  
 ATGCAGGTTACGGCTCCGGCTCACATTATACTGACGGATGCTTCAGTCACGTATCTTGC 1860  
 M Q V T A P A Q I N L T D A S V N V S C 600  
 GATTATAATGGTCTATGGTACCATTCAGCCANTGGAAGATGTTGGTTCTGAGTT 1920  
 D Y N G A I T I S A N G K M F G S A V 620  
 GTCGAAATTGCAACGCTACAATCAACTGACAGGCTGACAAATGAAACGCCGCTTAC 1980  
 V E N G T A T I N L T G L T N E S T L T 640  
 CTTAACAGTAGTTGGTACAAACAAAGAGACGGTAAAGACCATCAACACTAATGGTGAG 2040  
 L T V G Y N K E T V I K T I N T N G E 660  
 CCTAACCCCTACCAAGCCGGTTCCAACTTGACAGCTACAAACGCCAGGGTCAAGAGTAACG 2100  
 P N P Y Q F V S N L T A T T Q G O K V T 680  
 CTCAACTGGGATGACCCGAGCACGAAAACCAATGCAACCTAAATACCGCTCGCAGCGTG 2160  
 L K W D A P S T K T H A T T A R S V 700  
 GATGGCATACGAGAAATGGGTTCTCTGAGTCAGTCAGCGGATGCCCGGAACTTCTTCGGAGC 2220  
 D G I R E L V L S V S D A P E L L R S 720  
 GGTCAAGCCGAGATTTCTCTGAGCTACCGATGTTGGAAATGATGGATCCGGTTATCAG 2280  
 G Q A E I V L E A H D V W N D G S G Y Q 740  
 ATTCTTTCGGMTGCAACCATGATCAAATGAGCAGGTATACCCAGTGATACCCATACT 2340  
 I L L D A H D Q Y C Q V I P T D H T 760  
 CTTCGGCCGAACTGTGTCGGCCAAATCTGTCGGCTCCGTTCCGAAATATAGTTGTCGG 2400  
 L W P N C S V P A N L F A P F E Y T V P 780  
 GAAAATGCAAGATCCCTCTGATCCACCAATATGAAATGGATGGTACTTCGATCCGGT 2460  
 S N A D P S C S P T N M I M D G T A S V 800  
 AATATAACCGCCGGAACTTATGACTTCTGCAATTGCTGCTCCAAAGCAGAAATGCAAAAGATT 2520  
 N I P A G T Y D F A I R A P Q A N A K I 820  
 TGGATTCGGCCGAGCAAGGACCGACGAAAAGAGATGATATGAGCTTGTGAGCCGGTAAAGAA 2580  
 N I A G Q G P T K E D D Y V F E A G K K 840  
 TACCATTCCTTATGAGAGAAGATGGGTAGCGGTAGTGGAACTGAAATGACTATAAGCGAA 2640  
 Y H F L M K R M G S G D G T E L T I S E 860  
 GGTGGTGGAAAGGGATAACACTATACTGCTCATCTGAGCGGACAGAAGATCAAGGAAGGT 2700  
 G G G S D Y T Y T V R D G T K I R E G 880  
 CTGACCCAAACGACCAACCGGATACCGGATGAGTGCACAAATCTCATGAGTATTCGGTA 2760  
 L T E T T Y R D A G M S A Q G S H E Y C V 900  
 GAGGTAGTACGGCAGCCGGCTATCTGGAGGTTTGTTGAGGATTAATTCCTGACCGGA 2820  
 E V R K Y A A G V S P K V C V D Y I P D G 920  
 GTGGCAGACGCTAACGGCTCAGAAGCCCTAACACGGCTGACAGTTGGAAAGACGATCACG 2880  
 V A D V T A Q K P Y T L T V W G K T I T 940  
 GTAACTTGGCAACGGGAAACGTTATGATCTAGCAGACATGAAAGCTGCTGCTGGCAGCCGGT 2940  
 V T C Q G B R K I Y D M N G R R L A A G 960  
 CGCACACACTGTTTACCGCTCAGGGCGGCTACTATGCACTCATGCTTGTCCGTTGAC 3000  
 R N T V V Y T A Q G G Y Y A V M V V V D 980  
 GGCAGAGCTTACGTAGAGAAACTCGCTGTAAGTAATCTGCTGGACTCGGAGACTTT 3060  
 G K S Y V E K L A V K \* 991  
 GTGAGACACTTTAAATAGGTCTGTAATGTC

【0021】上記の本発明酵素の分析に使用できる、本発明の酵素を特異的に認識する抗体は、本発明酵素を用い、例えばフロインド(Freund)の完全アジュバントを用いた公知の方法で作成することができる。当該抗体の取得方法を以下に概略すると次の通りである。

【0022】すなわち、前記のようにして調製した、精製酵素液(約1mg)を等量のフロインドの完全アジュバントと混和し、その懸濁液を家兎等の皮下に数ヶ所に渡って注射し、これを2週間間隔で2回繰り返す。その後、ブースターを1回行って抗血清を採取する。採取した抗血清を、硫安処理及びプロテインA-セファロース(ファルマシア社、スエーデン)カラムクロマトグラフィーすることにより、1gG分画として抗体を得ることができる。

【0023】また、本発明の酵素と特異的に結合する合成基質を市販の入手可能な合成蛍光基質から見いだすに

は、例えは次の如くすれば良い。すなわち、10μMの基質、5mMシステインおよび当該酵素を含む反応溶液(20mMリン酸緩衝液、pH7.5)を40°Cで10分間加温した後、10mMヨード酢酸溶液(pH5)を加えて反応を停止させ、励起波長460nm、蛍光波長380nmで4-メチルクマリルーアミド(AMC)の遊離を測定し、AMCを遊離する基質を本発明の酵素と特異的に結合する合成基質とすれば良い。

【0024】各種の合成蛍光基質を用いた時の同一条件下での分解活性、すなわち、本発明酵素の基質特異性を表3に示す。この結果から、本発明酵素に特異的に認識される基質としては、Z-Phe-Arg-MCA、Boc-Phe-Ser-Arg-MCA、Boc-Gln-Ala-Arg-MCA等が選ばれる。これらの基質はペプチド研究所(大阪)から購入可能である。

【0025】

表 3

基質 合成基質 (10 μM)	活性 (μmol/mg/min)	最大活性 (%)
Boc-Phe-Ser-Arg-MCA	16600	100
Boc-Gln-Ala-Arg-MCA	11600	70
Z-Phe-Arg-MCA	16400	99
Z-Arg-Arg-MCA	1300	8
Boc-Glu-Lys-Lys-MCA	0	0
Boc-Val-Leu-Lys-MCA	160	1
Arg-MCA	670	4
Lys-MCA	0	0
Leu-MCA	1500	9
Ala-MCA	170	1
Gly-Pro-MCA	2000	12
Lys-Ala-MCA	500	3
Suc-Leu-Leu-Val-Tyr-MCA	0	0
Suc-Ala-Ala-Pro-Phe-MCA	0	0
Suc-Ala-Pro-Ala-MCA	0	0
Suc-Gly-Pro-Leu-Gly-Pro-MCA	0	0
Suc-Gly-Pro-MCA	0	0

【0026】歯周領域に存在する上記のP.シンジバリスの產生する酵素活性は、例えば下記方法により当該酵素を特異的に認識する合成基質及び／又は当該酵素を特異的に認識する抗体等を用いて測定することができる。

【0027】歯周領域に存在する本発明の酵素の測定方法は、具体的には以下のようにして行うことができる。

【0028】(1) 被検液の採取；被検液を採取する被検者としては、全身的疾患を持たない軽度から高度の歯周炎を有する臨床患者が適当であり、被検部位はX線写真上で明らかな垂直性の歯槽骨の吸収が認められる歯周ポケットを選択する。

【0029】歯肉溝滲出液(GCF)の採取は、まず簡易防湿後、歯肉縁上ブラークを注意深く除去し、ペリオペーパーストリップス(Harco Electronics, Canada)を用いてGCFを採取する。採取は、3枚のペーパーストリップスを用いて30秒間ずつ連続的に行なう。GCF量は、2番目のペーパーストリップスをペリオトロン6000(Harco Electronics, Canada)にかけて単位時間あたりに滲出してくる液量を計測する。

【0030】(2) 酵素活性の測定；予め氷冷していた300 μlのリン酸緩衝生理食塩水液(pH 7.4)に3枚のペーパーストリップスを浸し、0°Cで5～6時間放置した後、5分間の超音波処理を行い、遠心清を試料液として使用する。まず、被検液の一部を用い、これに一定量の基質(例えば、Z-Phe-Arg-MCA、Boc-Phe-Ser-Arg-MCA等)およびシステインを加え、40°C程度で加温した

後、ヨード酢酸溶液等を加えて反応を停止させる。ついで、励起波長460 nm、蛍光波長380 nmで4-メチルクマリルーザアミド(AMC)の遊離を測定し、上記基質に対する分解活性から酵素活性を求めることができる。

【0031】この時、EDTA(10 mM)及びロイペプチド(50 μM)の存在で阻害される活性量が目安としての当該酵素量となる(図3参照)。しかし、この段階での酵素量は、使用する基質が当該酵素のみによって認識されるものではなく、それ以外のプロテアーゼによっても分解を受ける可能性がある。従って、更に、被検液の一部に当該酵素に対する特異抗体を加え、37°Cで10分間反応させた後、反応液を遠心分離して固相(免疫複合体)と液相に分離し、液相中の上記基質に対する分解活性を測定して固相へ移行した活性量を算出すれば、より正確な当該酵素量が決定されることになる。

【0032】従って、より正確な酵素活性を求めるためには次の工程をとれば良い。

(1) 試料の一部を、EDTA及びロイペプチド存在下で本発明酵素の基質と反応させ、酵素活性を測定する、(2) 当該試料の一部に本発明酵素に対する抗体を作用させた後、当該反応液を固相と液相に分離し、次いで上記(1)に従って酵素活性を測定する、(3) 工程1の酵素活性より工程2の酵素活性を差し引き、本発明酵素活性量を算出する。

【0033】また、本発明の酵素をより簡便に測定するためには、(a) 特異的に請求項1記載の酵素と結合

する抗体、(b) 請求項1記載の酵素に特異的に認識される基質、(c) ロイペプチドおよび(d) EDTAを含む歯周病原性菌由来酵素測定用キットを利用すれば良い。

【作用】本発明は、後記表4に示すように、本発明酵素がP.ジンジバリスに特有のものであることに基づくものである。そして、歯周病患者の歯周ポケットにおける本発明酵素の活性上昇と歯周病の症状との関係は、以下の如くである。すなわち、歯周炎患者の歯肉溝から採取した滲出液中の当該酵素量は、単位時間あたりの滲出液量の増加(ペリオトロン値の増加)に比例して増大することが分かる(図4参照)。つまり、滲出液量が軽微な段階での当該酵素活性量は極めて低いが、滲出液量が増加するにつれてその活性量は増大し、中等度の症状ではその量は著しく増大し、重度になるとさらに増加することが判る。

【0034】滲出液量の増加と歯周炎の病状との間には相関関係があると一般に報告されていること(Cimasoni G.: Crevicular Fluid Updated. In: Myers HM, ed. Monographs in Oral Sciences. Basel, Karger, pp.1-152, 1983)や滲出液量と他の臨床パラメーター(歯肉指数や歯垢指数)との間にも相関関係があることを考えると、上記の結果は歯周病原性菌P.ジンジバリスの產生する当該酵素の活性量と歯周病の病勢との間には相関関係があることを示している。従って、本発明の方法を用いて得られた歯周病原性菌由来の酵素活性の測定値に基づいて、各歯周病患者における病状についてのより客観的判断が可能となるのである。

【0035】また、当該酵素は既に知られているP.ジンジバリス由来の50kDaシステインプロテアーゼ(ジンジペイン(gingipain); Chen Z, Potempa J, Polanowski A, Wikstrom M, Travis J: Purification and characterization of a 50-kDa cysteine proteinase (gingipain) from Porphyromonas gingivalis. J. Biol. Chem. 267:18896-18901, 1992)と至適pHやインヒビターに対する感受性等の酵素学的性質において類似しているが、基質特異性や熱安定性等の性質においては相違し、また、当該酵素は公知の上記酵素では明らかにされていない以下のような歯周病に密接に関係した極めて重要な性質を有しており、新規酵素であることが明らかである。

【0036】すなわち、タイプIコラーゲンや免疫グロブリン等の蛋白質をよく分解すること(図5参照)、セルビンやシスタチン等の重要な生体由来のプロテアーゼインヒビターによって阻害を受けにくくこと、多形核白血球の機能を濃度依存性、時間依存性に抑制すること(図6及び図7参照)、また、血清型を異にするP.ジンジバリスの複数の株には共通して存在しているが、他の歯周病原性菌といわれる細菌や腸内細菌の培養上清には存在しない(表4参照)等、当該酵素がP.ジンジバリス特有の歯周病原性因子として歯周組織の直接破壊や生体の防御系の破壊等に重要な役割を持つことが明らかにされている。従って、歯周病患者の歯肉溝滲出液中の本発明酵素を測定することは歯周病の診断において有用である。

【0037】

表 4

培養上清	プロテイン分解活性 (%)	
	Z-Phe-Arg-MCA	Boc-Phe-Ser-Arg-MCA
P. ジンジバリス 381	100.0	100.0
ATCC 33277	60.8	78.0
W50	90.4	99.1
SU63	56.9	63.6
14018	91.4	98.2
1112	66.4	70.3
GAI 7802	72.7	82.8
P. インターメディア ATCC 25611	0.2	0.2
P. メラニノゲニア ATCC 25845	0.1	0.1
B. フラギリス RIMD 0230001	0.2	0.2
ATCC 25285	0.3	0.9
A. アクチノマイセテムコミタンス ATCC 29522	0.3	0.2
ATCC 29523	0.5	1.0
S. ミュータンス 6715	0.9	0.9
S. サングイス ATCC 10557	0.2	0.2
E. コリ W3350	0.2	0.1
S. チフィムリウム B2245	0.1	0.1
ブレイン・ハート・インフュージョンプロス (対照)	0.2	0.2
トリプティケース・ソイプロス (対照)	0.0	0.0

【0038】表4は、血清型を異にするP.ジンジバリスの複数の株の培養上清及び他の歯周病原性菌といわれる細菌や腸内細菌の培養上清に含まれる当該酵素活性量を2種の合成基質を用いて測定した結果を示すものであるが、本発明酵素がP.ジンジバリス特有のものであることが示されている。

【0039】従って、本発明に係る酵素は上記のような酵素学的性質を有することから、当該性質を指標として、通常ヒト口腔内に存在するP.ジンジバリスの培養上清から精製を行うことにより、目的とする上記酵素を得ることができる。

## 【0040】

【実施例】以下、実施例により本発明を詳細に説明する。

## 【0041】実施例 1

P.ジンジバリス由来酵素の精製および酵素学的性質：本発明の酵素は以下のようにして精製した。P.ジンジバリス381株の培養上清に硫酸を加えて70%飽和とした。沈殿を遠心によって集め、非イオン性界面活性剤の0.05%ブリッジ35を含む10mMリン酸緩衝液(A液)に対して透析した。遠心透析上清をA液で平衡化したDEAEセファセルカラムにかけ、得られ

る非吸着分画を濃縮した後、さらにA液で平衡化したC M-トヨパールカラムにかけた(図8)。

【0042】カラムを同緩衝液でよく洗浄した後、当該酵素活性画分を70mM食塩を含む同緩衝液で溶出した。溶出画分は濃縮、透析の後、pH3.5~1.0の範囲の等電点分離にかけた(図9参照)。活性画分(pH5.0~5.5)を集め、濃縮、透析後、0.1M Na<sub>2</sub>SO<sub>4</sub>を含む10mMリン酸緩衝液で平衡化したTS

KゲルG2000SWのゲル汎過を行って精製した(図10参照)。

【0043】歯周病原性菌P.ジンジバリス由来酵素の精製方法のプロトコールと、基質としてZ-Ph e-A rg-MCAを用いた時の活性量(全活性と比活性等)の変化を表5に示す。

【0044】

表 5

工 程	プロテイン (mg)	全ユニット (U, ×10 <sup>-3</sup> )	特異活性 (U/mg)	收 率 (%)	倍 数
硫安分画	1270	1450	1140	100	1
DEAE-	188	1130	6000	78	5
セファセル					
CM-トヨパール	27	259	9600	18	8
650S					
等電点分離	11	168	15300	12	13
TSKゲル G	7	116	16600	8	15
2000SW					

#### 【0045】実施例 2

抗体の作成と特異性：本発明の酵素の測定方法において使用する抗体は、以下のようにして作成した。P.ジンジバリスの培養上清から精製した当該酵素液(約1mg)を等量のフロインド(Freund)の完全アジュバントと混和し、その懸濁液を家兔の皮下に数ヶ所に渡って注射し、これを2週間間隔で2回繰り返した。その後、ブースターを1回行って抗血清を採取した。

【0046】抗血清は硫安処理及びプロテインA-セファローズ(Protein A-Sepharose)(ファルマシア社、スエーデン)カラムクロマトグラフィーによってIgG分画として測定に供した。抗体の特異性は、対照として用いた当該酵素を含まないフロインド完全アジュバントのみを投与した家兔から得たIgG分画が当該酵素活性を全く中和しないことやウエスタンブロッティングでの非反応性によって確認される。また、当該酵素に対する抗体は他の歯周病原性細菌の培養上清のいかなるプロテアーゼ活性も阻害しないことから、極めて特異性の高い有用なものである。

#### 【0047】実施例 3

酵素の測定方法：本発明のP.ジンジバリス由来の酵素の測定方法は以下のようにして行った。まず、基質としてZ-Ph e-A rg-MCA又はBoc-Ph e-Ser-A rg-MCAを用い、被検溶液にこの基質溶液(10μM基質/5mMシステイン/20mMリン酸緩衝液、pH7.5)を加え、40°Cで10分間インキュベーションする。反応を同量の10mMヨー

ド酢酸溶液(pH5)を加えて止め、遊離したAMC量を蛍光分光光度計を用いた蛍光測定(励起波長380nm、蛍光波長460nm)によって決定する。この時、EDTA(10mM)及びロイペプチド(50μM)の存在下で同様の測定を行い、いずれの物質でも阻害される活性量が当該酵素量となる。

【0048】更に、被検溶液の一部に当該酵素に対する特異抗体を加え、37°Cで10分間反応させた後、反応液を遠心分離して固相(免疫複合体)と液相に分離し、液相中の上記基質に対する分解活性を測定して固相へ移行した活性量を算出すれば、より正確に当該酵素量を測定することができる。

#### 【0049】

【発明の効果】本発明は客観的且つ簡便な歯周病原性菌由来酵素の測定方法を提供するものである。本発明の酵素の測定方法を用いることにより、従来、客観的に診断することが困難であった歯周病の進行状況と活動度を経験に依存することなく的確に把握することが可能となり、歯科領域の治療法において大きく貢献するものである。また、本発明の酵素に対する抗体は、分析用試薬の他、本発明酵素の有する多形核白血球の反応抑制効果を阻害することからみて(図11参照)、歯周病の予防、治療剤としての利用も期待できる。

#### 【0050】

【配列表】配列番号：1

配列の長さ：991

配列の型：アミノ酸

トポロジー：

配列の種類：ペプチド  
配列

【化3】

TTTATGCATAAATACAGAAGGGTACTACACAGTAAATCATATTCTAAATTTCATCAA	60
ATGAAAAACITGAAACAAAGTTTGATGCTCCTTCCTTATTAGGAGGAATG	120
M K N L N K F V S I A L C S S L L G O M	20
GCATTGCGCAGCACAGACTTGGGACCCAACTCCGAATGTCAGATTCCTCGAAATCCACT	180
A F A Q Q T E L G R N P N V R L E S T	40
CAGCAATCGGTGACAAGGTTCACTTCCTGATGGACACCTCAAGTTAACCGAAGTCAA	240
Q Q S V T K V Q P R K D N L K F T E V Q	60
ACCCCTAAGGGATGGCACAAGTCCGACCTATACAGAAGGGTTAACCTTCCCAGAAA	300
T P K G M Q V P T Y T E G V N L S E K	80
GGGAATGCCCTACGCTTCCCATTCACGGCTCTTNGCGGTTTCAGACACTCGTGAGATG	360
G M P T L P I L S R S L A V S D T R E M	100
AAGGTAGAGGTTGTTCTCAAGTTCATCGAAAAGAAAATGTCCTGATTGCAACCTCC	420
K V E V V S S K Z I E K K N V L I A P S	120
AAGGGCATGATATGGTACGAGATCGGAAAGATCCCTACGGTTATGGAAAGAGC	480
K G M I M R N E D P K K I P X V Y G K S	140
TACTGGCAAAACAATCTTCCCGGAGAGATCGCACCCTTGATGATCCCTTATCCCT	540
Y S Q N K F F P G E I A T L D P F I L	160
CGTGTGTCGCTGGACAGGTGTAACACCTTGGCCCTTTCAGTATAACCTGTGACAAAG	600
R D V R G Q V V N F A P L Q Y N P V T K	180
ACGTTCGCACTATATGGAAATCACTTGGCAGTGAGCGAAACTTCGGAACAAGGCAA	660
T L R I Y T E I T V A V S E T S E Q G X	200
AATATTCTGACAGAAGGZACATTCGGCTTGTGAGACACATACAAAGGCATGTC	720
N I L N K G T F A G E F S D T Y K R M F	220
ATGAACTACCGAGCGGGCGTTACACACCGTAGAGGAAAACAAAATGGCTGATGATC	780
M N Y E P G R Y T P V E E K Q N G R M I	240
GTCATGTTAGCCAAAAGTATGAGGAGATTTAAAGATTCTGATTTGAAAACCAA	840
V I V A K K X E G D I K D F V D W K N Q	260
CGCGGCTTCGGTACCGAGGTGAAAGTGGCAGAAGATATGCTCTCCGTTACAGCTAA	900
R G L R T E V K V A E D I A S P V T A N	280
GCTATTCAGCAGGTTCTAACGAGAAAGAGAAGTAAATGATTTGACCTTATGTT	960
A I Q Q F V K Q E Y K E G N D L T Y V	300
CTTTGGTGGCGATCACAAAGATATTCTGGCTGGTCAAGGAAATCTCGGGGATCAAATCCGAC	1020
L L V G D H K D I P A K I T P G I K S D	320
CAGGTATATGGACAAATAGTAGGTAAATGACCACTACAAGAACCTTCATCCGTGCTTC	1080
Q V Y G Q I V G N D H Y N E V F I I G R F	340
TCATGTGAGAGCAAGGAGTCGAAGAACACATCGATCGGACTATTCACATATGAGCGC	1140
S C E S K E D L K T Q I D R T I H Y E R	360
AATAATACACCGGAAGACAAATGGCTGGTCAAGGAACTTCCTGGCTGAAGGA	1200
N I T T E D K W L G Q A L C I A S A E G	380
GGGCCATCCGCAAGAACATTGGTGAAGGTATTCAGCATGAGAACTTAATGCCAATCTG	1260
G P S A D N G E S D I Q H E T N E V F I I G R F	400
CTTACCCGATATGGCTATACCAAGATTATCAAAAGTTATGATCCGGACTAACCTCAA	1320
L T Q Y G Y T K I I K C Y D F G V T P K	420
AACATTAATGAGCTTCACGGAGGAATCTGGTCAACIATACGGGCCACCGTAGC	1380
N I I D A F N G G I S L V N Y T G H G S	440
GAAACAGCTTGGGTAAGTCTCACTTGGCACCCTCTAGTGAAGGAGCTTACCAACAGC	1440
E T A U G T S H F G T T H V K Q L T N S	460
AACCAAGCTACCGTTTATTCGACGCTGCTGTGCAATGGCGATTTCCTATTCAGCATG	1500
N Q L F F I F D V A C V N G D F L F S M	480
CCTTGCTTCGCAAGACCGCTGATGGTGCACAAAGATGGTAAGCGACAGGTACTGTT	1560
P C P A E A L M R A Q K D G K P T G T V	500
GCTATACCGCTCTACGATCAACCACTCTGGCTCTTCCTATGGCGCCCGAGGATGAG	1620
A I I A S T I N Q S W A S P M R G Q D E	520
ATGAAOGAAATTCGTGGCAAAACACCCGAACAAACATCAAGGCTACTTCGGTGGTGC	1680
M N E I L C E K H P N N I K R T F G G V	540
ACCATGAAACGGTATGGTGTATGGTGGAAAAGTATAAAAAGGATGGTGAAGAGATGCTC	1740
T M N G M F A M V E K Y K K D G E K M L	560

【化4】

GACACATGGACTTTCCGGCAGCCCTCGCNGCTGCTGCTTGTACACTTGCTCCGACCAA 1800  
 D T W T V F G D P S L L V R T L V P T K .580  
 ATGCAGGTAAAGGCTCCGGCTCAGATAATTGACGGATGCTTCAGTCACGTAATCTGCC 1860  
 M Q V T A P A Q I N L T D A S V N V S C 600  
 GATTATAAATGGTCAATTGCTACCAATTCAAGGAAAGATGCTTCGGTCTGCAAGTT 1920  
 D Y N G A I A T I S A N G K M F G S A V 620  
 GTCCAATATGCAACAGCTAACATCAATCTGACAGGCTGACAAATGAAACGACGGCTTAC 1980  
 V E N G T A T I N L T G L T N E S T L T 640  
 CTTACAGTAGTTGGTCAACAAAGAGACGGTTATTAGACCATCAACACTAATGGTGAG 2040  
 L T V V G Y N K E T V I K T I N T N G E 660  
 CCTAAACCCCCTACCAAGCCCCGTTTCCAACCTGACAGCTACCGCAGGTACAGAAAGTAAACG 2100  
 P N P Y Q P V S N L T A T T Q G Q K V T 680  
 CTCAACTGGATGCCACCGAGCACGAAAACCAATGCAACCAATPACCCCTGCCAGCGT 2160  
 L K W D A P S T K T N A T T N T A R S V 700  
 GATGGCATACGAGARATGGTCTCTGTCAGTCAGCGATGCCCGGACTCTCTGCCAGC 2220  
 D G I R E L V L L S V S D A P E L L R S 720  
 GGTCAAGGCCGAGATTGCTCTGAAGCTCACGATGTTGGAAATGATGGAAACCGGTTATCAG 2280  
 G Q A E V L E A D V W N D G S G Y Q 740  
 ATTCTTCTGGATGCAACGACCATGATGATGATGAGCTTACCCAGTGATACCCACACT 2340  
 I L L D A H D Q G V Q V I P S D T H T 760  
 CTTPGCCGAAACTGTAATGTCGGOCATACTGTTCCGCTCGGAAATATACTGTTGG 2400  
 L W N P V S P A N L F A P Y T V P 780  
 GAAAATGCAAGATCTCTGTTCCCTACCAATAATGATAATGGATGGTACTTGCACTCCGGT 2460  
 S N A D P S C S P T N M I M D G T A S V 800  
 AATATAACCGCCGGAACCTTACCTGCTTCAATTGCTGCTCTCACAGGAAATGCCAAAGATT 2520  
 N I P A G T Y D F A I A A P Q A N A K I 820  
 TGGATITGCGGACAGGACGACGAAAGAGATGATGATGTTGAAGCCGGTAAAGAAA 2580  
 W I A G O Q G P T K E D D Y V F E A G K K 840  
 TACCAATTCTCTTATGAGAAGAGATGGTAGCGGTGATGGAACCTGAACTTATAAGCGAA 2640  
 Y H F L M K G M S G D G T E L T I S E 860  
 GGTGGTGGAAAGGGATTAACCTTACTACTCTCTATGGTCAAGGCGACAGAATGGAAAGGT 2700  
 G G G S D Y T Y T V Y R D G T K I K E E G 880  
 CTGACCGGAAACGACCTTACCGCGATGCAAGGAATGAGTGACACAATCTCAAGGATTTGGCTA 2760  
 L T E T Y T R D A G M S A Q S H E Y C V 900  
 GAGGTAAAGTACGGCAGCCGGCTATCTCCGAAGGTTGGTCTGGATTATACTCTGACCGA 2820  
 E V K Y A A G V G S P K V C V D Y I P D G 920  
 GTGGCAAGACGTAACGGCTCAGAACGCTTACACGCTGACAGTTGGAAAGACGATCACG 2880  
 V A D V T A Q K P Y T L T V W G K T I T 940  
 GTAACCTGCGCAAGGGAAACCTTATGATCTACGACAGAACGGCTCGCTGCGCAGCCGGT 2940  
 V T C Q G E R M I Y D M N G R R L A A G 960  
 CGCAACACAGTGTGTTACACGGCTCAGGGCGCTACTATGCACTGCTTGTGTTGAC 3000  
 R N T Q V V Y T A Q O G Y Y A V M V V V D 980  
 CGCAAGTCTACGTAGAGAAACTCGCISTAAAGTANTCTGCTTGGACTCGGAGACTT 3060  
 G R S Y V E K L A V R \* .991  
 GTGCAAGACACTTAAATAGGCTCTGAAATGTC 3094

## 【図面の簡単な説明】

【図1】 本発明酵素が2種類の合成基質を分解する際のpHの影響を示す図面。

【図2】 本発明酵素の熱安定性を示す図面。

【図3】 歯周病原性菌、P. ジンジバリス培養上清の有する多形核白血球のケイルミネッセンス反応抑制作用に対する、各種プロテアーゼインヒビターの作用を示す図面。

【図4】 歯周病患者の歯肉溝滲出液中に含まれる本発明酵素量を示す図面。測定は基質としてBoc-Phe-Ser-Arg-MCAを用いた。図中1ユニットは、40°Cにおいて、1分当たり $1\mu\text{mol}$ のAMCを遊離させるのに必要な量を意味し、ペリオトロン値の1ユニットは、GCF 0.005μlに対応する。

【図5】 本発明酵素によるヒト由来コラーゲンタイプI(lane 2, 3)およびタイプIV(lane 4, 5)の分解パターンを示す図面。各コラーゲンを当該酵素と20°C(A)または37°C(B)で10時間インキュベーションした後、SDSゲル電気泳動を行った。レーン1は分子量マーカー、レーン2および4は当該酵素非存在下、レーン3および5は当該酵素(1μg)存在下でそ

れぞれ処理をした。

【図6】 精製された本発明酵素(5μg)を用いて多形核白血球(2×10<sup>6</sup>個)のケミルミネッセンス反応に対する抑制効果を経時的に調べた結果を示す図面。

【図7】 本発明酵素による多形核白血球のケミルミネッセンス反応抑制効果に対する濃度依存性を示す図面。

黒丸は当該酵素に1mMシスティンを加えたもの、白丸は酵素のみを加えたもの、黒三角は酵素に1mMシスティンと40μg/mlのロイペプチドを加えたものを示す。

【図8】 本発明酵素のCMトヨパールでのゲル汎過パターンを示す図面。

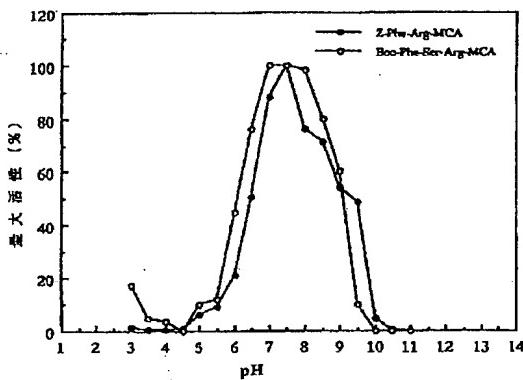
【図9】 本発明酵素のカラム等電点電気泳動での分布パターンを示す図面。

【図10】 精製された本発明酵素のゲル電気泳動パターンを示す図面。単一のバンドが見られることから、当該酵素は均一にまで精製されていることが分かる。

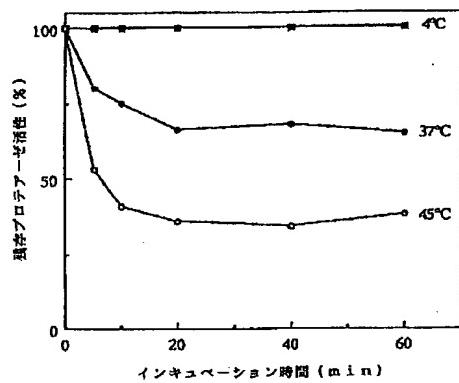
【図11】 本発明酵素に対する特異抗体が当該酵素の有する多形核白血球のケミルミネッセンス反応抑制効果を阻害することを示す図面。

以上

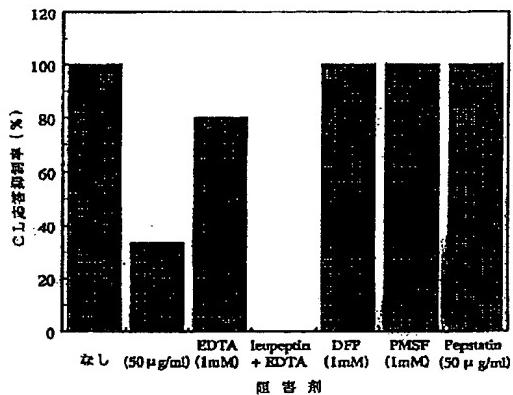
【図1】



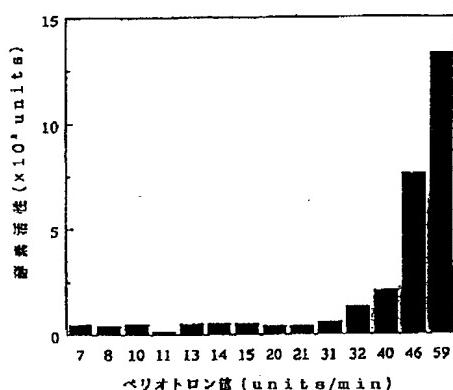
【図2】



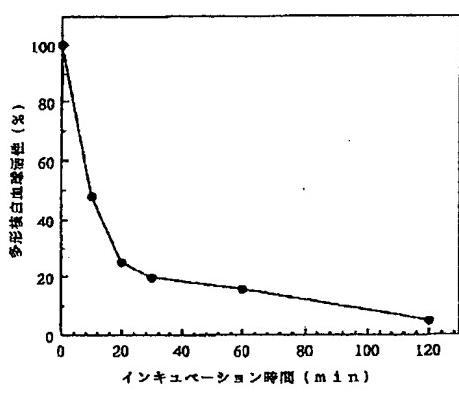
【図3】



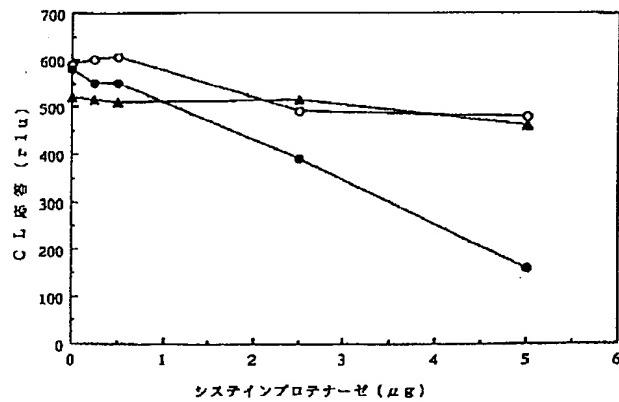
【図4】



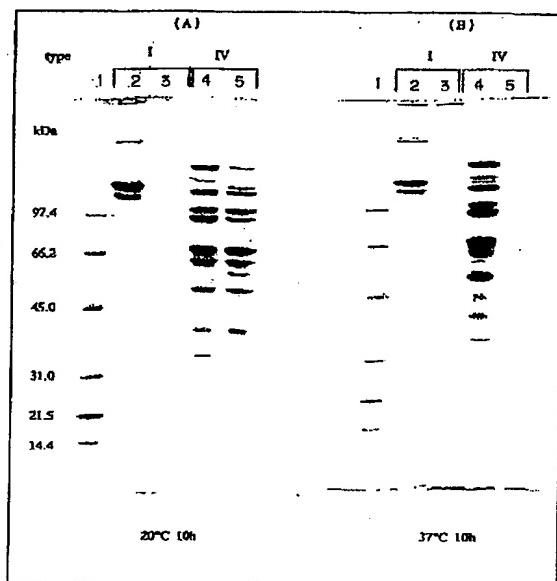
【図6】



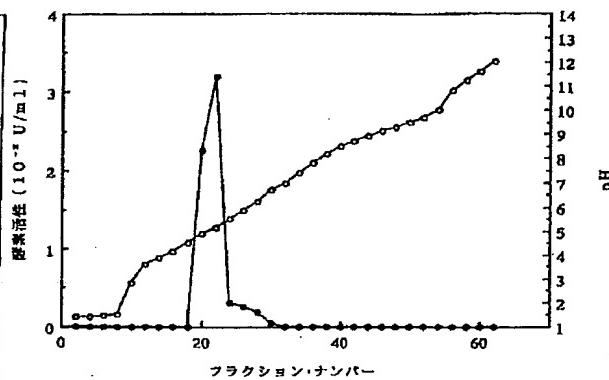
【図7】



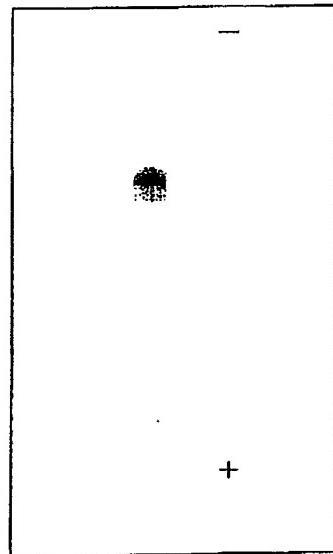
【図5】



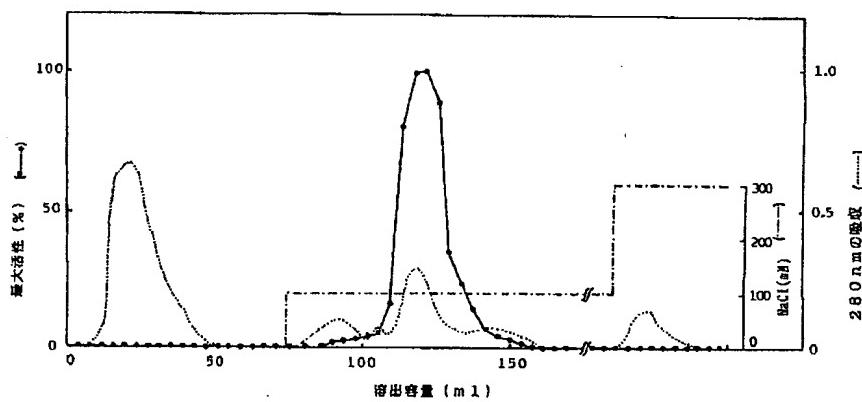
【図9】



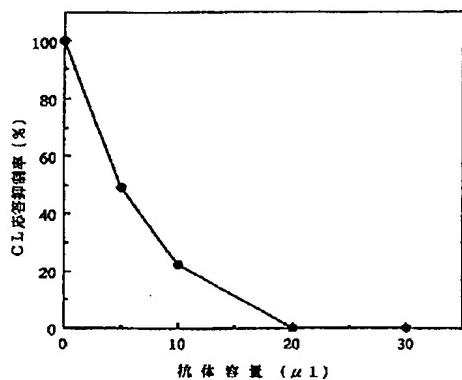
【図10】



【図8】



【図11】



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フロントページの続き

(51) Int.Cl.<sup>6</sup>  
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技術表示箇所

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## DETAILED DESCRIPTION

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### [Detailed Description of the Invention]

#### [0001]

[Field of the Invention] This invention relates to the new protease in which a code is carried out by the new protease gene of the Bacteroides forcer ISASU (Bacteroides forsythus) origin which is one of the organisms of a periodontal disease, and this gene. Furthermore, this invention relates to the vaccine containing this protease, etc.

#### [0002]

[Description of the Prior Art] Periodontosis is one of the two major diseases in the mouth, and is a compound infectious disease by several sorts of bacteria. In it, since it separates into high frequency from the part of progressive periodontitis, Bacteroides forcer ISASU is considered to be one of the main organisms of adult nature periodontitis or recurrent periodontitis. In order that bacteria may produce protease outside a biomass, these attract attention as a pathogen factor. Therefore, the molecular biology approach about this protease is not solved at all in molecular biology about the protease which contributes to a break through of the generating mechanism of periodontosis, etc. and which starts although it is [ however ] large.

[0003] On the other hand, as a preventive measure of periodontosis, Porphyromonas gingivalis (Porphyromonas gingivalis) and Actinobacillus actinomycetemcomitans (Actinobacillus.) which are the bacteria which participate in periodontosis as well as Bacteroides forcer ISASU All the actinomycetemcomitans biomasses, the biomass extract, or the vaccine that uses a pilus as an antigen is proposed (JP,S59-128338,A, JP,S61-140527,A, JP,H8-176014,A). The proposal is made also about the vaccine which uses the pilus and capsule of Bacteroides forcer ISASU as an antigen (JP,H5-132428,A).

[0004] However, these had a problem in respect of that the fall of the characteristic of a vaccine takes place with impurity, and safety. Then, about Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans. The vaccine which uses as an antigen peptide of the amino acid sequence origin of the protein which constitutes the pilus is proposed (JP,H8-48695,A, JP,H9-52846,A). However, there is no influence of impurity about Bacteroides forcer ISASU, and the vaccine which can moreover expect a high effect is not known.

#### [0005]

[Problem(s) to be Solved by the Invention] The purpose of this invention Therefore, the Bacteroides forcer ISASU origin, DNA which encodes the polypeptide which has protease activity and hemolysis activity, It is in providing the vaccine against the manufacturing method of polypeptide and this polypeptide and Bacteroides forcer ISASU which have the protease activity and hemolysis activity of the Bacteroides forcer ISASU origin, and the diagnostic drug of periodontosis.

#### [0006]

[Means for Solving the Problem] Namely, a gist of this invention, [1]DNA which is DNA which may be obtained from a chromosomal DNA of Bacteroides forcer ISASU (Bacteroides

forsythus), and encodes polypeptide which has protease activity and hemolysis activity, [2]The above whose Bacteroides forcer ISASU is 43037 shares of Bacteroides forcer ISASU ATCC [1]DNA of a description, [3]An array number of an array table: DNA which consists of a base sequence shown in one, [4]An array number of an array table: DNA which has a part of base sequence shown in one, and encodes polypeptide which has protease activity and hemolysis activity, [0007][5]An array number of an array table: DNA which are DNA which consists of a base sequence shown in one, and DNA hybridized under stringent conditions, and encodes polypeptide which has protease activity and hemolysis activity, [6]An array number of an array table: DNA which 1 or some bases are deletion and DNA added, inserted or replaced, and encodes polypeptide which has protease activity and hemolysis activity in a base sequence shown in one,[7]An array number of an array table: DNA which encodes polypeptide which consists of an amino acid sequence shown in two, [8]An array number of an array table: DNA which is DNA which encodes polypeptide which has a part of amino acid sequence shown in two, and encodes polypeptide which has protease activity and hemolysis activity, [0008][9]An array number of an array table: DNA which is DNA which encodes polypeptide which 1 or some amino acid residue become from deletion and an amino acid sequence added, inserted or replaced in an amino acid sequence shown in two, and encodes polypeptide which has protease activity and hemolysis activity, [10]Above[1]-[9]It is the polypeptide by which a code is carried out to DNA of a description either, [11]An array number of an array table: Polypeptide which consists of an amino acid sequence shown in two, [12]An array number of an array table: Polypeptide which 1 or some amino acid residue are deletion and the polypeptide added, inserted or replaced, and has protease activity and hemolysis activity in an amino acid sequence shown in two, [0009][13]An array number of an array table: Polypeptide which is the polypeptide which has a part of amino acid sequence shown in two, and has protease activity and hemolysis activity, [14]Above[1]-[9]It is an expression vector in which it comes to insert DNA of a description either, [15]Above[14]A cell into which it comes to introduce an expression vector of a description, [16]Above[15]A manufacturing method of polypeptide which has protease activity and hemolysis activity collecting polypeptides which have protease activity and hemolysis activity which are acquired by culturing a cell of a description, [17]Above[10]-[13]It is an antibody specifically combined with polypeptide of a description, or its part either, [0010][18]The above which is peptide fragments in which a part of polypeptide consists of five or more continuous amino acid residue [17]An antibody of a description, [19]The above which has the activity which mediates an individual and derives protective immunity to Bacteroides forcer ISASU [10]-[13]It is a vaccine containing polypeptide of a description, or its part either, [20]The above which is peptide fragments in which a part of polypeptide consists of five or more continuous amino acid residue [19]A vaccine of a description, [21]Above[10]-[13]It is a diagnostic drug containing polypeptide of a description, or its part of a disease by Bacteroides forcer ISASU either, [0011][22]The above which is peptide fragments in which a part of polypeptide consists of five or more continuous amino acid residue [21]A diagnostic drug of a description, [23]Above[1]-[9]It is a diagnostic drug containing DNA of a description, or its part of a disease by Bacteroides forcer ISASU either, [24]Above[17]or[18]It is related without a diagnostic drug of a disease by Bacteroides forcer ISASU containing an antibody of a description.

[0012]

#### [Embodiment of the Invention]

(A) DNA of this invention will not be limited in particular for DNA of this invention, if it is DNA which encodes protease outside a biomass of the Bacteroides forcer ISASU origin. In this Description, 43037 shares of Bacteroides forcer ISASU [ 293 shares of ] ATCC, FDCs [ 291 shares of ], and FDC etc. are mentioned as a desirable thing as Bacteroides forcer ISASU.

Specifically as a DNA of this invention, the following DNAs can be illustrated.

[0013]1) DNA which is DNA which may be obtained from the chromosomal DNA of Bacteroides forcer ISASU, and encodes the polypeptide which has protease activity and hemolysis activity..

2) The array number of an array table : DNA which consists of a base sequence shown in one.

3) The array number of an array table : DNA which encodes the polypeptide which consists of an amino acid sequence shown in two.

[0014]DNA which encodes the polypeptide which is DNA which has a part of base sequence shown in array number:1 of an array table about DNA of 2), and has protease activity and hemolysis activity, The array number of an array table : DNA which encodes the polypeptide which are DNA which consists of a base sequence shown in one, and DNA hybridized under stringent conditions, and has protease activity and hemolysis activity, And the array number of an array table: In the base sequence shown in one, 1 or some bases are deletion and DNA added, inserted or replaced, and DNA which encodes the polypeptide which has protease activity and hemolysis activity is also contained in DNA of this invention.

[0015]DNA which consists of a base sequence shown in array number:1 of an array table here is DNA obtained from the chromosomal DNA of Bacteroides forcer ISASU, and is an open reading frame of the polypeptide which has protease activity and hemolysis activity. In this Description, "a part of base sequence" says 5-1271 bases, for example.

[0016]Saying "it hybridizes under stringent conditions." As a hybridization buffer, 5xSSC, 5x DENHARUTO, 0.1%SDS and a 250microg/mL salmon sperm DNA content 50mM phosphoric acid buffer (pH 6.5) are used, It says hybridizing on the conditions that hybridization temperature performs 1 hour and 2xSSC by 50 \*\* and 6xSSC (room temperature), and performs washing for 5 minutes by SDS (50 \*\*) 0.1%. "Some" means 1-5 pieces about the deletion of a base, addition, insertion, or the number of substitution, for example.

[0017]It is DNA which encodes the polypeptide which has a part of amino acid sequence shown in array number:2 of an array table about DNA of 3, In DNA which encodes the polypeptide which has protease activity and hemolysis activity, and the amino acid sequence shown in array number:2 of an array table, 1 or some amino acid residue are DNAs which encode the polypeptide which consists of deletion and an amino acid sequence added, inserted or replaced, and DNA which encodes the polypeptide which has protease activity and hemolysis activity is also contained in DNA of this invention.

[0018]Here, the amino acid sequence shown in array number:2 of an array table is an amino acid sequence in which a code is carried out by the base sequence shown in array number:1, and is an example of the amino acid sequence of new protease outside a biomass of the Bacteroides forcer ISASU origin.

[0019]The polypeptide by which a code is carried out to DNA of this invention has protease activity and hemolysis activity. The part of the polypeptide of this invention explains the explanation about this activity about polypeptide.

[0020]Cloning of the DNA of this invention can be carried out by producing a genomic library by a publicly known method from Bacteroides forcer ISASU, and screening this library. For example based on the basic document of Molecular Cloning 2nd Ed. (Cold Spring Harbor Laboratory Press (1989)) etc., a person skilled in the art can perform cloning easily. The decision of the base sequence of DNA by which cloning was carried out can be made by the publicly known method of using a commercial sequence kit etc. For example by a site-directed-mutagenesis method etc., if it is a person skilled in the art, it can perform easily introducing variation, such as deletion, addition, insertion, or substitution, into an amino acid sequence or a base sequence.

[0021]DNA (protease gene) of such this invention encodes the protease which attracts

attention as a pathogen factor of periodontosis, and is useful because of the diagnosis, the therapy, and research concerning periodontosis.

[0022](B) The polypeptide of this invention will not be limited in particular for the polypeptide of this invention, if it is protease outside a biomass obtained from *Bacteroides forcer* ISASU and is the polypeptide which has protease activity and hemolysis activity. Here, as *Bacteroides forcer* ISASU, 43037 shares of *Bacteroides forcer* ISASU [ 293 shares of ] ATCC, FDCs [ 291 shares of ], and FDC etc. are mentioned as a desirable thing. Specifically as polypeptide of this invention, the following polypeptides can be illustrated.

[0023]1) Polypeptide which is the polypeptide by which a code is carried out to DNA which may be obtained from the chromosomal DNA of *Bacteroides forcer* ISASU, and has protease activity and hemolysis activity.

2) The array number of an array table : polypeptide which consists of an amino acid sequence shown in two.

3) The array number of an array table : polypeptide by which a code is carried out to DNA which consists of a base sequence shown in one.

[0024]In the amino acid sequence shown in array number:2 of an array table about the polypeptide of 2), 1 or some amino acid residue are deletion and the polypeptide added, inserted or replaced, It is the polypeptide which has protease activity and hemolysis activity, and the polypeptide which has a part of amino acid sequence shown in array number:2 of an array table, and the polypeptide which has protease activity and hemolysis activity is also contained in the polypeptide of this invention.

[0025]In this Description, "some" means 1-5 pieces about the deletion, addition, insertion, or substitution of amino acid residue, for example. In this Description, "a part of amino acid sequence" says five or more residue, for example. The molecular weight of the polypeptide of the amino acid sequence shown in array number:2 to this invention is calculated from about 48 kDa(s) and calculation.

[0026]It is the polypeptide by which a code is carried out to DNA which has a part of base sequence shown in array number:1 of an array table about the polypeptide of 3), It is the polypeptide by which a code is carried out to the polypeptide which has protease activity and hemolysis activity, DNA which consists of a base sequence shown in array number:1 of an array table, and DNA hybridized under stringent conditions, In the polypeptide which has protease activity and hemolysis activity, and the base sequence shown in array number:1 of an array table, 1 or some bases are the polypeptides by which a code is carried out to deletion and DNA added, inserted or replaced, and the polypeptide which has protease activity and hemolysis activity is also contained in the polypeptide of this invention.

[0027]The polypeptide of this invention has protease activity and hemolysis activity. In this Description, say "hemolysis activity" and the activity which acts on erythrocyte membrane, collapses this and separates hemoglobin this activity, Specifically in accordance with the method (*Microbiology and Immunology* (96) 40: 717-723) of Kimizuka and others, it can measure.

[0028]namely, the polypeptide or the polypeptide inclusion of a measuring object -- a phosphoric acid buffer (0.8-% of the weight sodium chloride.) It melts in a 0.02-% of the weight calcium chloride, 0.115-% of the weight phosphoric acid 1 hydrogen sodium (anhydrous), and 0.02-% of the weight potassium dihydrogen phosphate (anhydrous) (pH 7.5), and the sample solution is prepared. Subsequently, the amount mixing of isochore of the blood and this sample solution of *Homo sapiens* who did centrifugal washing with the phosphoric acid buffer beforehand is carried out, and it settles at 37 \*\* for 1 hour. The absorbance of 541 nm is measured about the supernatant liquid of mixed liquor after settlement. Saponin liquid (the phosphoric acid buffer solution of saponin (saponin by Nacalai Tesque, Inc.), 0.1 % of the weight of concentration of saponin) and the phosphoric acid buffer

perform respectively same operation instead of the sample solution, and each absorbance of 541 nm is measured. An absorbance when a phosphoric acid buffer is used from an absorbance when saponin liquid was used is lengthened, and let the value which hung 0.5 on the value be 1 hemolysis activity unit (HU). Let what has polypeptide or the activity of 1 or more HU per mg of polypeptide inclusion be "the polypeptide or the polypeptide inclusion which has hemolysis activity."

[0029]"Protease activity" can mean the activity which hydrolyzes a peptide bond, and it can measure by the following methods in this Description. That is, the inclusion of the polypeptide of a measuring object or polypeptide is dissolved in 50mM tris buffer (pH 8.0), and the sample solution is prepared. Subsequently, synthetic substrates, such as this sample solution and N-benzoyl-Val-Gly-Arg-p-nitroanilide, and the tris buffer are mixed, and it incubates at 37 \*\* for 1 hour. And the absorbance of 405 nm is measured about the mixture after incubation. What used the tris buffer instead of the sample solution is considered as contrast. Suppose there "there is protease activity" that out of which the value higher than the absorbance of contrast came. The extracts (for example, bacillus surface extract etc.) of the transformant which DNA which encodes the polypeptide of this invention, for example comes to contain as an inclusion of the polypeptide of a measuring object are mentioned about measurement of hemolysis activity and protease activity.

[0030]The polypeptide of this invention is protease which has the following character. The publicly known protease activity measuring method using the synthetic substrate of substrate specificity versatility shows that the polypeptide of this invention is what has the substrate specificity shown in Table 1.

[0031]

[Table 1]

合成ペプチド	活性 (%)± SD	
	実施例	対照
N-Benzoyl-Val-Gly-Arg- pNa	100	0.4±0.2
BAPNA	1.5±0.7	1.3±0.1
L-Phe-Ala-pNa	2.7±0.2	3.0±0.6
Gly-Phe-pNa	2.9±0.4	2.6±0.04
Gly-Pro-pNa	0.3±0.5	0.1±0.1
Ala-Ala-Phe- pNa	1.6±0.3	1.4±0.3
N-Suc-Gly-Gly-Phe- pNa	2.0±0.1	1.1±0.5
N-Suc-Ala-Ala-Ala- pNa	2.1±0.4	1.3±0.6
N-Suc-Ala-Ala-Pro-Phe- pNa	1.4±0.5	0.9±0.5
N-Suc-Ala-Ala-Pro-Leu- pNa	1.4±0.1	1.2±0.1
N-Benzoyl-L-Tyro-pNa	1.0±0.03	1.3±0.3
Glu-L-Phe- Ala-pNa	0.5±0.04	0.6±0.2
N-Benzoyl-Pro-Phe-Arg- pNa	1.1±0.2	0.3±0.1
N-p-Tosyl-Gly-Pro-Arg- pNa	1.2±0.3	1.4±0.6

[0032]As for the polypeptide of influence this invention given by various protease inhibitor, the protease activity is checked by TLCK, leupeptin, N-ethyl malei mide, iodoacetic acid, an iodoacetamide, and EDTA. This shows that this polypeptide is a cysteine protease.

[0033]The polypeptide of this invention can be obtained by collecting the polypeptides which have the protease activity and hemolysis activity which are acquired by culturing the cell into which the expression vector in which DNA of this invention was inserted was introduced. The

produced polypeptide can be easily refined with the refining method of publicly known protein, such as affinity refining which used the antibody of the usual column chromatography or below-mentioned this invention, and can be collected.

[0034]Although publicly known vectors, such as pET, pCDM8, and pBluescript SKII+, are mentioned for example, the vector used for construction of the expression vector of this invention can insert DNA of this invention, and especially if it is a vector which can be revealed, it will not be limited. The method in particular of inserting DNA of this invention in a vector is not limited, and can use the method that the method of using T4 DNA ligase, etc. are publicly known. The cell of this invention which is a transformant is obtained by introducing said expression vector into a desired host cell. As a host cell, any of a prokaryote cell or an eukaryote cell may be sufficient, and it is chosen according to the expression vector to be used. What is necessary is just to use publicly known methods, such as a calcium phosphate method, the CaCl<sub>2</sub> method, the DEAE dextran process, and the electroporation method, as a method of introducing an expression vector, for example.

[0035]The polypeptide of such this invention is useful because of the diagnosis, the therapy, and research concerning periodontosis.

[0036](C) As long as it is an antibody combined specifically [ the antibody of this invention ] about the antibody of this invention to the polypeptide of this invention, or its part, any of a polyclonal antibody or a monoclonal antibody may be sufficient. This antibody is easily producible in accordance with the method indicated, for example to JP,H7-97395,A by carrying out immunity of a rabbit, the mouse, etc. using all or a part of polypeptides of this invention. As a use of this antibody, screening of affinity chromatography and a cDNA library, medicine, a diagnostic drug, a laboratory reagent, etc. are mentioned.

[0037]The polypeptide for production of an antibody can be refined from the culture of a transformant in which DNA of this invention was introduced, and peptide which is a part of polypeptide can be obtained by a publicly known peptide synthesis method based on the amino acid sequence of the polypeptide of this invention.

[0038]It will not be limited especially if the antibody to this fragment combines with this polypeptide or its part specifically as peptide fragments which consist of a part of polypeptide. The peptide fragments which consist of five or more continuous amino acid residue in the polypeptide of this invention as a part of polypeptide, for example are mentioned. The peptide fragments which have an amino acid sequence in the range of the 1st place - the 377th place of the amino terminal of the amino acid sequence shown, for example by array number:2 as such peptide fragments are preferred, and the peptide fragments which have an amino acid sequence in the range of the 1st place - the 190th place are more preferred. The peptide fragments which have an amino acid sequence shown in array number:3 of an array table - array number:5 as these peptide fragments, for example are mentioned.

[0039]In this invention, the derivative of the peptide fragments described below, the salt of peptide fragments, or the salt of this derivative can be used as peptide fragments as a part of polypeptide used for production of an antibody, or a vaccine and a diagnostic drug. That is, in this invention, the derivative of the derivative of the following peptide fragments and peptide fragments, the salt of peptide fragments, or the salt of this derivative is included by a part of polypeptide.

[0040]In this Description, the derivative of peptide fragments refers to what combined a protective group, a functional group, a spacer, or a carrier with peptide fragments. For example, that from which the amino terminal of peptide fragments was protected by acetyl group or a urethane group, and the thing from which the C terminal was protected by amide group or an ester group are the examples of the derivative which has a protective group. What introduced tyrosine and cystein into peptide fragments is included by the derivative. When an antibody combines with peptide fragments, a spacer may be introduced the making steric

exclusion hard to receive purpose, but that into which such a spacer was introduced is also included by the derivative.

[0041]As a derivative which the carrier combined, what polylysine, various polymer, bovine serum albumin, tetanus toxoid, ovalbumin, keyhole limpet hemocyanin, thyroglobulin, gamma globulin, a polysaccharide, etc. combined is included.

[0042]As the salt of peptide fragments, or a salt of a peptide-fragments derivative, When the peptide fragments themselves or its derivative has an amino group, The salt of it and inorganic acid with a salt formation operation, such as chloride, sulfuric acid, phosphoric acid, and pyrophoric acid, Furthermore, acid addition salt, such as a salt with organic acid, such as acetic acid, lactic acid, pulmitic acid, stearic acid, propionic acid, citrate, tartaric acid, malic acid, ascorbic acid, oxalic acid, methanesulfonic acid, benzenesulfonic acid, and p-toluenesulfonic acid, is mentioned.

[0043]On the other hand, when the peptide fragments themselves or its derivative has a carboxyl group and a sulfonyl group, salts, such as alkaline earth metal salt, such as alkali metal salt, such as those sodium salt and potassium salt, calcium salt, and magnesium salt, ammonium salt, and a triethylamine salt, are mentioned.

[0044]The derivative of peptide fragments can be obtained by the chemical modification means usually used by the technical field of peptide chemistry. For example, when carrying out the chemical bond of the carrier to peptide fragments, the method of using glutaraldehyde, a water-soluble carbodiimide, a succinimide, etc. can be used.

[0045](D) About the vaccine of this invention, the vaccine of this invention comes to contain the polypeptide of this invention of a description, or its part in the above as an active principle, and has the activity which medicates an individual and derives the protective immunity to Bacteroides forcer ISASU. The same thing as the peptide fragments indicated in the part of explanation of the antibody of this invention as peptide fragments which consist of a part of polypeptide used for the vaccine of this invention is mentioned as a desirable thing.

[0046]Water, salting in liquid, glucose, or glycerin may be blended with the vaccine of this invention as another constituent, for example. Bile acid, its derivative or those salts, a surface-active agent, a cholera toxin B subunit, etc. may be blended with a vaccine as absorption enhancers.

[0047]As other ingredients, alum, aluminium hydroxide, aluminium phosphate, Adjuvants, such as aluminum sulfate and a muramyl dipeptide; Oleic acid, Parts for adjuvant fat fusing, such as stearic acid and pulmitic acid; Sorbic acid, Chlorobutanol, benzoic acid, a paraoxybenzoic acid, boric acid, Antiseptics, such as dehydroacetic acid and Timor; Sodium polyacrylate, polyvinyl alcohol, A polyvinyl pyrrolidone, carboxymethylcellulose sodium, methyl cellulose, Binders, such as hydroxyethyl cellulose, a carrageenan, sodium alginate, gum arabic, xanthan gum, montmorillonite, kaolin, hydration silica, magnesium aluminum silicate, and hectorite, can be blended. As a medication method of a vaccine, methods, such as subcutaneous injection, an intramuscular injection, injection in the mouth, an intravenous injection, nasal administration, internal use, and oral cavity mucosal administration, are mentioned.

[0048]The constituent for the mouths containing the specific antibody contained in the blood serum, the cow's milk, and the egg which are produced by carrying out immunity of the polypeptide of this invention or its part to a cow or a hen can be used for periodontosis prevention or a therapy as what is called passive immunity. Although a typical first time dose is 0.1 - 1.0 mg/kg weight as a protein amount, according to the grade of the prevention or the therapy to need, it increases a dose or should just increase frequency of administration.

[0049](E) DNA of the polypeptide of this invention or its part, and this invention or its thing which contained the antibody of this invention in part can be used as a diagnostic drug of diseases, such as periodontosis, about the diagnostic drug of the disease of this invention. For

example, when the diagnostic drug containing the polypeptide of this invention or its part is used, The polypeptide of this invention or its part is fixed in a polystyrene plate, a glass filter, a magnetic particle, latex, etc., and periodontosis can be diagnosed using the ELISA method, the RIA method, a fluorescent antibody technique, chemiluminescence antibody technique, etc. which detect a test subject's specific antibody by a sign second antibody. The same thing as the peptide fragments indicated in the part of explanation of the antibody of this invention as peptide fragments which consist of a part of polypeptide used for the diagnostic drug of this invention is mentioned as a desirable thing.

[0050]When using the diagnostic drug containing DNA of this invention, or its part, periodontosis can be diagnosed using the Southern blot technique using this DNA or its DNA obtained from the saliva of test subject origin, a dental plaque, gingival crevice fluid, etc. in part as a probe, etc.

[0051]

[Example]Hereafter, although working example explains this invention in more detail, this invention is not limited at all by these working example.

[0052]Working example 1 (cloning of a protease gene)

Cloning of the protease gene of Bacteroides forcer ISASU was performed in accordance with the method (Infection and Immunity (95) 63: 1147-1152) of Ishihara and others. That is, the chromosomal DNA was settled by ethanol after extraction with phenol from 43037 shares of Bacteroides forcer ISASU ATCC. Partial digestion of the obtained chromosomal DNA was carried out by restriction enzyme Sau3A.I. Artificial Intelligence, and the DNA fragment of 2 - 10kbp was obtained according to 10 to 40% of sucrose density gradient centrifugation (154000xg, 18 hours). Those fragmentation was included in plasmid vector pBluescript SKII+, and this expression vector was introduced into Escherichia coli HB 101 by the CaCl<sub>2</sub> method. Escherichia coli HB 101 transformed was grown by LB agar medium which contains skim milk and 60microg/mL ampicillin 1% of the weight, and what made the macula pellucida around the colony was made into the positive clone.

[0053]SK primer is made into a primer by using as a mold the plasmid obtained from a positive clone, Using a die primer sequence kit (made by Applied Biosystem), by the JIDIOKISHI nucleotide chain termination method. The base sequence was determined by the automatic DNA sequencer (the product made by Applied Biosystem, model 373A).

[0054]Thus, the base sequence of the open reading frame of protease outside a biomass of obtained Bacteroides forcer ISASU is shown in array number:1 of an array table. The amino acid sequence of the polypeptide by which a code is carried out to the base sequence shown in array number:1 is shown in array number:2.

[0055]Working example 2 (production of recombination protease)

The positive clone obtained in working example 1 was cultivated with LB broth containing 60microg/mL ampicillin. The obtained biomass was centrifuged after ultrasonic crushing and supernatant liquid was obtained. From this supernatant liquid, protein was condensed with the sedimentation method by ammonium sulfate. From the obtained protein, using the Lot Foix cell (made by Bio-Rad Laboratories), the activity fraction was obtained and partial purification of the protease was carried out by isoelectric focusing. From the activity fraction, it rearranged using the Superdex 200 HR gel-filtration (made by Pharmacia) column, and protease was refined.

[0056]Next, it investigated about the protease activity and substrate specificity of polypeptide of this invention of the transformed Escherichia coli origin. Bacillus surface extract 50muL of transformed Escherichia coli HB 101, synthetic substrate 50microL shown in Table 1, and 50mM tris buffer (pH 8.0) 150microL were incubated at 37 \*\* for 1 hour, and the absorbance of 405 nm of the mixture was measured. A result is shown in Table 1. In Table 1, the activity value to each synthetic substrate showed the absorbance of N-benzoyl-Val-Gly-Arg-p-

nitroanilide as 100 (%).

[0057]It experimented similarly about the bacillus surface extract of Escherichia coli HB 101 which is not transforming. This data was considered as contrast. As a result, it turned out that the protease which carried out cloning is protease specific to N-benzoyl-Val-Gly-Arg-p-nitroanilide.

[0058]The influence which various protease inhibitor has was investigated about the polypeptide contained in the bacillus surface extract of transformed Escherichia coli HB 101. As a result, as for this polypeptide, the protease activity was checked by TLCK, leupeptin, N-ethyl malei mide, iodoacetic acid, an iodoacetamide, and EDTA. This showed that this polypeptide was cystein pro TEASE.

[0059]Ultrasonic crushing of the Escherichia coli HB 101 transformed was carried out, and the bacillus surface extract was obtained. thus, obtained bacillus surface extract 400μL and a phosphoric acid buffer (0.8-% of the weight sodium chloride.) A 0.02-% of the weight calcium chloride, 0.115-% of the weight phosphoric acid 1 hydrogen sodium (anhydrous), And blood 400μL of Homo sapiens and a horse which carried out centrifugal washing with potassium dihydrogen phosphate (anhydrous) (pH 7.5) 0.02% of the weight was incubated at 37 \*\* for 1 hour, and the absorbance of 541 nm of the supernatant liquid of a mixture was measured. instead of [ of a bacillus surface extract ] -- saponin liquid (the phosphoric acid buffer solution of saponin (saponin by Nacalai Tesque, Inc.)) 0.1 % of the weight of concentration and the phosphoric acid buffer of saponin performed respectively same operation, the absorbance when the phosphoric acid buffer was used from the absorbance when saponin liquid was used was lengthened, and the value which hung 0.5 on the value was made into 1 hemolysis activity unit (HU).

[0060]As a result, the bacillus surface extract of transformed Escherichia coli HB 101 showed hemolysis activity (14.2 HU/mg and 10.8 HU/mg) to Homo sapiens and a horse, respectively. The bacillus surface extract of Escherichia coli HB 101 which has not been transformed used as contrast does not show activity to which blood, It turns out that the polypeptide of this invention which is protein of the DNA origin introduced into the bacillus surface extract of transformed Escherichia coli HB 101 from it having been 0 HU/mg contains. It is possible that it is participating in periodontitis by this hemolysis activity.

[0061]Working example 3 (production of an antibody)

In accordance with the method of a description, MULTI-PIN PEPTIDE SYNTHESIS KIT made from CHIRON MIMOTOPES PTY LTD (Australia) is used for JP,H8-48695,A, Three kinds of peptide which consists of 5-15 amino acid residue based on the amino acid sequence of the polypeptide drawn from the base sequence of DNA of this invention obtained in working example 1 was compounded from the amino terminal side. the compounded peptide -- respectively -- the peptide 1 (an amino acid sequence -- array number: -- shown by three.), it being considered as the peptide 2 (an amino acid sequence -- array number: -- shown by four.) and the peptide 3 (an amino acid sequence -- array number: -- shown by five.), and, respectively -- array number: -- it corresponds to the 44-48th place (peptide 1) of the amino acid sequence shown by two, the 168-177th place (peptide 2), and the 363-377th place (peptide 3).

[0062]In accordance with the method of by TSUKAICHISU and others (Vaitukaitis) (J. Clin. Endoch. 33.988 (1971)), the above-mentioned peptide, It emulsified by Freund's complete adjuvant and 3 times subcutaneous injection of the peptide of the quantity of 100microper mouse weight of 1 kg g was carried out to five BALB/c mice of five weeks old every two weeks. Blood was extracted one week after the last immunity, and the existence of the antibody production was investigated by the ELISA method.

[0063]Measurement by the ELISA method was performed as follows. 43037 shares of Bacteroides forcer ISASU [ 100micro of ] ATCC were prepared to the concentration of g

(lyophilized cell)/mL, and the biomass was fixed on 96 hole microtiter plate, and it blocked continuously. Subsequently, the liquid which diluted the extracted blood was added to the well. The well was washed after neglect for 60 minutes at 37 \*\*, and the alkaline phosphatase sign sheep antimouse immunoglobulin antibody was added. The well was washed after neglect for 60 minutes at 37 \*\*, subsequently to a well the hydrogen peroxide solution (30%) of the 2 chloride o-phenylenediamine (0.04 % of the weight) of 200microL, and 10microL/100mL was added, and the absorbance of 490 nm was measured. The mouse which injected only Freund's complete adjuvant instead of peptide was processed similarly, and blood was extracted. This thing was considered as contrast. A result is shown in Table 2.

[0064]

[Table 2]

アミノ酸配列	吸光度
SPDRTW	0. 580
YI IDDDLMSA	0. 602
NGTTNNNSKLMKITDA	0. 621
対照	0. 1以下

[0065]Table 2 showed that a specific antibody was produced by Bacteroides forcer ISASU by a part of polypeptide of this invention. From this, it was shown that the polypeptide of this invention or its part can be used as a vaccine.

[0066]Working example 4 (use as a vaccine)

The polypeptide of this invention or some of its initial complements are taken, it fully mixes with the incomplete Freund's adjuvant of the amount of isochore, or a complete Freund's adjuvant, and the vaccine formulations of an oil Nakamizu system are obtained.

[0067]Working example 5 (use as a diagnostic drug)

The peptide compounded in working example 3 was fixed on 96 hole microtiter plate in accordance with the conventional method. The blood serum of the periodontosis patient and the healthy person was diluted with the phosphate buffer solution, and it added to the well which fixed peptide. After washing a well, it incubated at 37 \*\* [ anti-human IgG antibody / commercial / peroxidase labelling rabbit ] as a second antibody. Subsequently, after washing a well, o-phenylenediamine solution was added to the well and the absorbance of 405 nm was measured. What added equivalent weight of PBS instead of the diluent of a blood serum, and performed same operation to it was made "sample additive-free." A result is shown in Table 3.

[0068]

[Table 3]

アミノ酸配列	吸光度	
	歯周病患者	健常者
SPDRTW	0. 628	0. 264
YI IDDDLMSA	0. 607	0. 351
NGTTNNNSKLMKITDA	0. 674	0. 298
検体無添加	0. 1以下	0. 1以下

[0069]Table 3 showed that the difference of the absorbance between a healthy person and a periodontosis patient was remarkable. Therefore, it turned out that peptide of this invention can be used as a diagnostic drug of periodontosis.

[0070]Working example 6 (Southern hybridization using DNA of this invention)

Partial digestion of 43037 shares of Bacteroides forcer ISASU ATCC, three shares of clinical isolates, and Porphyromonas gingivalis was carried out by Sau3A.I. Artificial Intelligence (as control), and they were given to agarose gel electrophoresis. The tris buffer neutralized and the nylon filter was made to transfer, after carrying out alkaline denaturation of the migration gel in 1.5M sodium chloride / 0.5N sodium hydroxide solution.

[0071]The plasmid probe which carried out the label to this nylon filter by the DIGOKISHI genin of 1 - 10pmol was made to hybridize, and it incubated at 42 \*\* overnight. It added and the anti-DIGOKISHI genin alkaline phosphatase conjugate antibody was made to color after washing a nylon filter using the late-coming color reagent which incubated.

[0072]The plasmid probe was produced as follows. DIG DNA The label of plasmid vector pBluescript SKII+ in which DNA which encodes the polypeptide of this invention by DIG-dUTP was inserted was carried out using the labeling system (made by Boehringer Mannheim). This thing was made into the plasmid probe.

[0073]Hybridization was carried out as follows. 42 \*\* incubated for 18 hours with a 50% formamide, 5xSSC, the 1% blocking buffer, the 0.1% sarcosine, and the hybridization buffer that contains SDS 0.01%. DIG DNA after detection washing the hybridized nylon filter It carried out using the detection kit (made by Boehringer Mannheim).

[0074]As a result, 43037 shares of Bacteroides forcer ISASU ATCC and three shares of clinical isolates formed the band in the position of 0.6kbp and 0.8kbp, respectively. A band was not looked at by Porphyromonas gingivalis. From this, detection of Bacteroides forcer ISASU and diagnosis of periodontosis found that DNA of this invention was useful.

[0075]

[Effect of the Invention]The polypeptide by which a code is carried out to DNA of this invention and this DNA is useful because of the diagnosis about periodontosis, a therapy, and research. The vaccine of this invention and a diagnostic drug are useful to prevention of periodontosis, a therapy, and diagnosis.

[0076]

[Layout Table]

array number: -- length [ of 1 arrangement ]: -- mold [ of 1272 arrangement ]: -- number [ of nucleic acid chains ]: -- double strand topology: -- kind [ of straight-chain-shape arrangement ]: -- Genomic DNA sequence: -- ATG GCT CCA ATG GGA GCT GTA TGG GAT GAC CGA TCT TTG GCG CTA 45Met. Ala Pro Met Gly Ala. Val Trp Asp Asp Arg. Ser Leu Ala Leu 1 5. 10 15 TCA TCT AAG ATG. GCT TTT GCC AAT GAG. AAG TTG AGA TAT CTG. TTT 90Ser Ser Lys Met. Ala Phe Ala Asn Glu. Lys Leu Arg Tyr Leu. Phe 20 25 30 TGG TCA. ACT TGT CTC AGT TTA. AGA GTC CAC GAT GGA. CAT TCT CCT 135Trp Ser. Thr Cys Leu Ser Leu Arg Val His Asp Gly His Ser Pro 35 40 45 GAT CGT ACC TGG CGA CTT GCC AAT AAA GGA GGG. TTG AGG ATG ATC 180Asp. Arg Thr Trp Arg Leu. Ala Asn Lys Gly Gly. Leu Arg Met Ile 50 55. 60 TTT GGT TAT GAA ACG. GTA TCT TAT GAC AGT. GGC CGC TAT GGC AGT. 225Phe Gly Tyr Glu Thr. Val Ser Tyr Asp Ser. Gly Arg Tyr Gly Ser. 65 70 75 GAA TTT TGG. AAA CAA TGG AAA AAA. GGA AAG AGT TTT TCT. GAT GCT 270Glu Phe Trp. Lys Gln Trp Lys Lys. Gly Lys Ser Phe Ser. Asp Ala 80 85 90 TTT. ATT GAA GCC AGT TGG. TCG CTA TTC CGG AAT CAA ACG CCC GTC 315Phe Ile Glu Ala Ser Trp Ser Leu Phe Arg Asn Gln Thr Pro Val 95 100 105 GTT TGT GCA T. GT GGC AAT ACT AAA GAG. GAG GTT CAA AAA AGG. CTG 360Val Cys Ala Cys. Gly Asn Thr Lys Glu. Glu Val Gln Lys Arg. Leu 110 115 120 TTT. TCG GAA AGA ATG TTC. TAT TCC GGG GCG GTG. AGC AGC AAC TGG 405Phe. Ser Glu Arg Met Phe. Tyr Ser Gly Ala Val. Ser Ser Asn Trp 125. 130 135 TAT TGG TGG. AAG TGG AGA GAG GCC. CAA AAC CAT AAA GGC. CTT AAG 450Tyr Trp Trp. Lys Trp Arg Glu Ala. Gln Asn His Lys Gly. Leu Lys 140 145 150. ACA GCA AGA GCG AAG GCC CCT

CAA AAT ATG GAT GTG TTG CTC TTG 495Thr Ala Arg Ala Lys Ala Pro Gln Asn Met Asp. Val Leu Leu Leu 155. 160 165 AAA CCG TAC. ATA ATT GAC GAT GAT TTG ATG TCA GCA ATT GCA AAT 540Lys Pro Tyr Ile Ile Asp Asp Asp Leu Met Ser Ala Ile Ala Asn 170 175 180 AAA GTC GGC ATC AAC AAA ATA. ACG GCA AAA TCG ATT. GCT ATC GGA 585Lys Val. Gly Ile Asn Lys Ile. Thr Ala Lys Ser Ile. Ala Ile Gly 185 190. 195 CAA GAT GGT TTA. CGT TGT ATC GGA ACA. AAA GAC ATT TTG GTA. AGT 630Gln Asp Gly Leu. Arg Cys Ile Gly Thr. Lys Asp Ile Leu Val. Ser 200 205 210 GTG. GAT TCT TCA GGG ACG. CTG CAA TTA CAA ATG. GCT CAG GCT AAT 675Val. Asp Ser Ser Gly Thr. Leu Gln Leu Gln Met. Ala Gln Ala Asn 215 220 225 TAT CGG AAT GAC AAC CGG ATC AGC GAA ATC CAA GCA ACC AAG ATT 720Tyr Arg Asn Asp As. n Arg Ile Ser Glu Ile. Gln Ala Thr Lys Ile. 230 235 240 GCC AGA. GAA TTG ATT GAA GAT. TTG GGA ATT GCC AAG. GAT GTC AAA 765Ala Arg. Glu Leu Ile Glu Asp. Leu Gly Ile Ala Lys. Asp Val Lys 245 250. 255 TTG ACA CCG GCA. ACT ACG TAT AAC TCG. TTC CTG TGT GGT GCC. AAT 810Leu Thr Pro Ala. Thr Thr Tyr Asn Ser. Phe Leu Cys Gly Ala. Asn 260 265 270 ACG. AAA ACT GGC GAG CAG. GGT AAG CCA ACG GTG. GTA GAG ACT ATT 855Thr. Lys Thr Gly Glu Gln Gly Lys Pro Thr Val Val Glu Thr Ile 275 280 285 ATT CAG TTC CGC CAA GTC AAT GAC AAA ATG. GAA AGT GTG AAT GCA. 900Ile Gln Phe Arg Gln. Val Asn Asp Lys Met. Glu Ser Val Asn Ala. 290 295 300 GAT TCC. GGT TTC GTT GCA GTC. GCT GTT GAC AAC GAC. GGA AAG ATC 945Asp Ser. Gly Phe Val Ala Val. Ala Val Asp Asn Asp. Gly Lys Ile 305 310. 315 ACA CGT CTA ACC. AGC TCC GTC AAA CCA. ATT GTA GAT ACC CAA. AAG 990Thr Arg Leu Thr. Ser Ser Val Lys Pro. Ile Val Asp Thr Gln. Lys 320 325 330 AGT. ATT GAT ATG CAG AGC. CTT GCT AAA AAG AGG GAG GTC AAA ATG 1035Ser Ile Asp Met Gln Ser Leu Ala Lys Lys Arg Glu Val Lys Met 335 340 34. 5 AAG GAG CTT TCT GTC. GAA GAA CGA TTC GAA. AGA AAG ATC AAT CGC. 1080Lys Glu Leu Ser. Val Glu Glu Arg Phe. Glu Arg Lys Ile Asn. Arg 350 355 360 TTG. ATA AAC GGA ACG ACA. AAC AAC AGC AAG TTG. ATG AAG ATA ACA 1125Leu. Ile Asn Gly Thr Thr. Asn Asn Ser Lys Leu. Met Lys Ile Thr 365. 370 375 GAT GCC CCA. AAG GTA ATG GTC GAA. ACA CTC TCC GAT AAA. ATC GGG 1170Asp Ala. Pro Lys Val Met Val. Glu Thr Leu Ser Asp. Lys Ile Gly 380 385 390 TAT GAC TTC TCT TCC AAC TAT GCC CAA CCG GTC CAG CAG CGT GAT 1215Tyr Asp Phe Ser Ser Asn T. yr Ala Gln Pro Val Gln. Gln Arg Asp 395 400. 405 ATC GAA ATC AAA. GTG GGC GAT TTT GTA. AAA CGC TAC CAA TTG. AGA 1260Ile Glu Ile Lys Val Gly Asp Phe Val Lys Arg Tyr Gln Leu Arg 410 415 420 GTA GAT TTG TAA 1272Val Asp Leu[0077]array number: -- length [ of 2 arrangement ]: -- mold [ of 423 arrangement ]: -- number [ of amino acid chains ]: -- single strand topology: -- kind [ of straight-chain-shape arrangement ]: -- protein array: -- Met Ala Pro Met Gly Ala Val Trp Asp Asp Arg Ser Leu Ala Leu 1 5 10 15. Ser Ser Lys Met Ala. Phe Ala Asn Glu Lys. Leu Arg Tyr Leu Phe. 20 25 30 Trp Ser Thr. Cys Leu Ser Leu Arg. Val His Asp Gly His. Ser Pro 35 40 45 Asp. Arg Thr Trp Arg Leu. Ala Asn Lys Gly Gly. Leu Arg Met Ile 50 55. 60 Phe Gly Tyr Glu Thr. Val Ser Tyr Asp Ser. Gly Arg Tyr Gly Ser. 65 70 75 Glu Phe Trp Lys Gln Trp Lys Lys Gly Lys Ser Phe Ser Asp Ala 80 85 90 Phe Ile Glu Ala Ser Trp Ser L. eu Phe Arg Asn Gln Thr. Pro Val 95 100 105 Val. Cys Ala Cys Gly Asn. Thr Lys Glu Glu Val. Gln Lys Arg Leu 110. 115 120 Phe Ser Glu. Arg Met Phe Tyr Ser. Gly Ala Val Ser Ser. Asn Trp 125 130 135. Tyr Trp Trp Lys Trp. Arg Glu Ala Gln Asn. His Lys Gly Leu Lys. 140 145 150 Thr Ala. Arg Ala Lys Ala Pro. Gln Asn Met Asp Val. Leu Leu Leu 155 160. 165 Lys Pro Tyr Ile. Ile Asp Asp Asp Leu. Met Ser Ala Ile Ala. Asn 170 175 180 Lys Val Gly Ile Asn Lys Ile Thr Ala Lys Ser Ile Ala Ile Gly 185 190 195 Gln Asp Gly Leu Arg Cys I. le Gly Thr Lys Asp Ile. Leu Val Ser 200 205. 210 Val Asp Ser Ser. Gly Thr Leu Gln Leu. Glu Met Ala Gln Ala. Asn 215 220 225 Tyr. Arg Asn Asp Asn Arg. Ile Ser Glu Ile Gln. Ala Thr Lys Ile 230. 235 240 Ala Arg Glu. Leu Ile Glu Asp Leu. Gly Ile Ala Lys Asp. Val Lys 245 250 255. Leu Thr Pro Ala Thr.

Thr Tyr Asn Ser Phe. Leu Cys Gly Ala Asn. 260 265 270 Thr Lys. Thr Gly Glu Gln Gly. Lys Pro Thr Val Val. Glu Thr Ile 275 280 285 Ile Gln Phe Arg Gln Val Asn Asp Lys Met Glu Ser Val Asn Ala 290 295 300 Asp Ser Gly Phe Val. Ala Val Ala Val Asp Asn Asp Gly Lys Ile 305 310 315 Thr Arg Leu Thr Ser Ser Val Lys Pro Ile Val Asp Thr Gln Lys 320 325 330 Ser Ile Asp Met Gln Ser Leu Ala Lys Lys Arg Glu Val Lys Met 335 340 345 Lys Glu Leu Ser Val Glu Glu Arg Phe Glu Arg Lys Ile Asn Arg 350 355 360. Leu Ile Asn Gly Thr. Thr Asn Asn Ser Lys. Leu Met Lys Ile Thr. 365 370 375 Asp Ala. Pro Lys Val Met Val. Glu Thr Leu Ser Asp. Lys Ile Gly 380 385. 390 Tyr Asp Phe Ser. Ser Asn Tyr Ala Gln. Pro Val Gln Gln Arg Asp 395 400 405 Ile Glu Ile Lys Val Gly Asp Phe Val Lys Arg Tyr Gln Leu Arg 410 415 420 Val Asp Leu[0078]array number: -- length [ of 3 arrangement ]: -- mold [ of 6 arrangement ]: -- number [ of amino acid chains ]: -- single strand topology: -- kind [ of straight-chain-shape 配列 :

Ser Pro Asp Arg Thr Trp  
arrangement ]: -- peptide 1 5

[0079]array number: -- length [ of 4 arrangement ]: -- mold [ of 10 arrangement ]: -- number [ of amino acid chains ]: -- single strand topology: -- kind [ of straight-chain-shape arrangement 配列 :

Tyr Ile Ile Asp Asp Asp Leu Met Ser Ala  
]: -- peptide 1 5 10

[0080]array number: -- length [ of 5 arrangement ]: -- mold [ of 15 arrangement ]: -- number [ of amino acid chains ]: -- single strand topology: -- kind [ of straight-chain-shape arrangement ]: -- peptide sequence: -- Asn Gly Thr Thr Asn Asn Ser Lys Leu MetLys Ile Thr Asp Ala 1 5 10 15

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[Translation done.]